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SECTION MEETINGS

CLEVELAND
Western Reserve University April 13, 1945

E. V. McCOLLUM

14957

Comparison of Changes in Serum Glucose, Serum Potassium and Blood Pressure After Epinephrine.*

Foster N. Martin, Jr. (Introduced by Ralph G. Smith.)

From the Departments of Pharmacology, University of Michigan and the Medical College of the State of South Carolina.

D'Silva[1] and Schwarz[2] described independently the effect of epinephrine in increasing serum potassium. Harrop and Benedict[3] had previously described a fall in serum potassium following insulin. They suggested that this fall in serum potassium was due to involvement of potassium in the formation of the phosphate carbohydrate compound. The fact that epinephrine increases the blood sugar as well as the serum potassium would seem to be support for this. The present work was undertaken to test this theory on the basis of the chronologic relationship of these actions.

Experimental. Cats were anesthetized with pentobarbital and atropinized. Blood samples were taken from a femoral artery before, and as rapidly as possible for the first two minutes after the injection of epinephrine. Further samples were taken at gradually increasing intervals up to periods of from one to 2 hours. Serum potassium was determined by the method of Hoffmann[4] using the Evelyn photoelectric colorimeter. Serum sugar was determined by a Folin micro method also involving the use of the photoelectric colorimeter. The carotid blood pressure was recorded with a mercury manometer. On the same record the time of blood sampling was also recorded. A dosage of 0.075 mg of epinephrine per kg of body weight by intravenous injection was used on 4 cats and in a fifth cat 0.025 mg per kg of epinephrine was used.

Results. The administration of 0.075 mg per kg of epinephrine gave essentially the same results on all animals. In Fig. 1 are recorded the average results obtained from the 4 cats. The same chronologic relationship between blood pressure and potassium changes occurred as previously reported when dosages

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* This work was presented as partial fulfillment of the requirements for the Ph.D. degree in Pharmacology at the University of Michigan.

[1] D'Silva, J. L., *J. Physiol.*, 1934, **82**, 393; 1936, **86**, 219.

[2] Schwarz, H., *Archiv. f. d. Exp. Path. u. Pharm.*, 1935, **177**, 628.

[3] Harrop, G. A., and Benedict, Ethel M., *J. Biol. Chem.*, 1924, **59**, 683.

[4] Hoffmann, W. S., *J. Biol. Chem.*, 1937, **120**, 57.

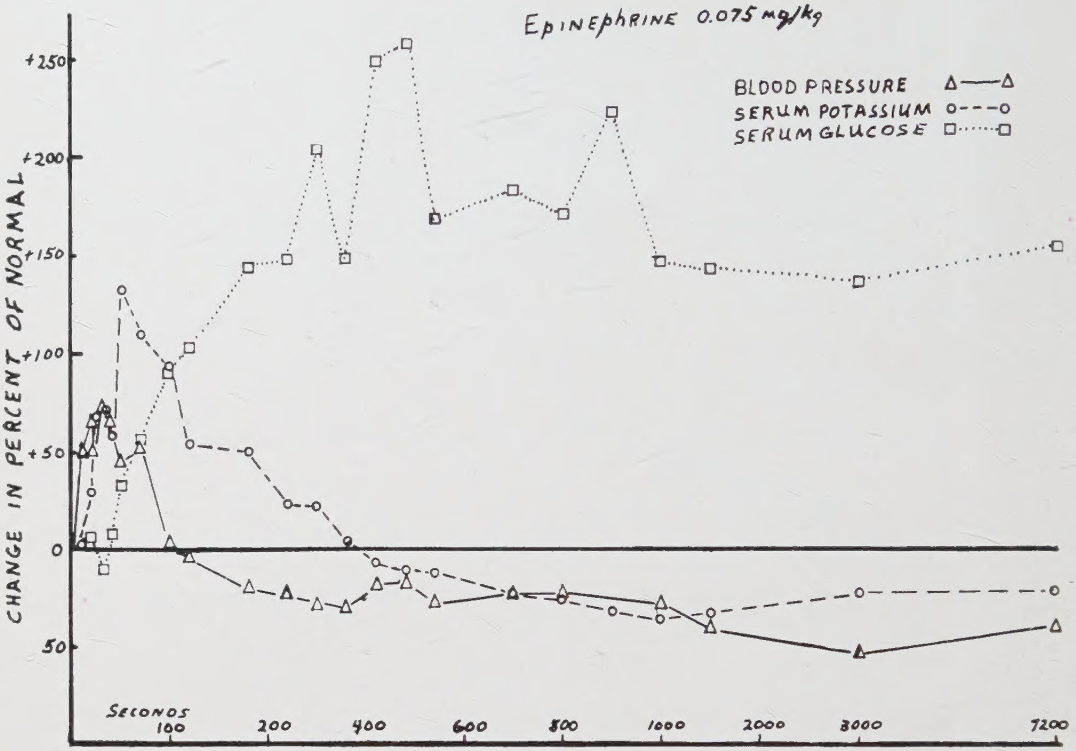

FIG. 1.

Average changes in blood pressure, serum potassium and serum glucose following the intravenous injection of epinephrine in cats.

of 0.1 and 0.05 mg per kg of epinephrine were used.[5] The potassium effect was later by many seconds and was proportionately greater than the blood pressure rise. The peak of the potassium rise occurred after the blood pressure had begun to fall and remained high after the blood pressure had fallen below normal. The rise in blood sugar was slower even than the potassium effect since the rise did not begin until 50 seconds after injection as compared with 20 seconds for potassium. The curve did not reach a maximum until after the blood pressure and potassium had returned to normal or below normal and was still elevated at the end of the experimental period. The results were qualitatively the same with a dose of 0.025 mg per kg of epinephrine (Fig. 2).

Discussion. The delay of the glucose effect suggests the possibility that some decomposition product of epinephrine is responsible for this action rather than the unaltered drug, as suggested by Bacq[6] for the inhibitory effects of epinephrine. Supplementary experiments were carried out in an attempt to test this suggestion. The "adrenoxine" of Bacq[6] (Bacq and Heirman)[7] has not been obtained in sufficient amounts to make its use in such an experiment feasible so attempts were made to produce adrenochrome by oxidation of epinephrine. Enzymatic oxidation of epinephrine (Green and Richter)[8] produces adrenochrome which may be crystallized as a pure compound. Two atoms of oxygen are used for each molecule of epinephrine when the process is carried out in a Warburg apparatus. Since the enzymes and apparatus for such oxidation were unavailable a cruder method of oxidation with potassium permanganate was tried. Attempts to isolate the

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[5] Martin, F. N., *J. Pharm. and Exp. Therap.*, 1942, **76**, 270.

[6] Bacq, Z. M., *J. Physiol.*, 1938, **92**, 28P.

[7] Bacq, Z. M., and Heirman, P., *Arch. Int. de Physiol.*, 1940, **50**, 129.

[8] Green, D. E., and Richter, D., *Biochem. J.*, 1937, **31**, 596.

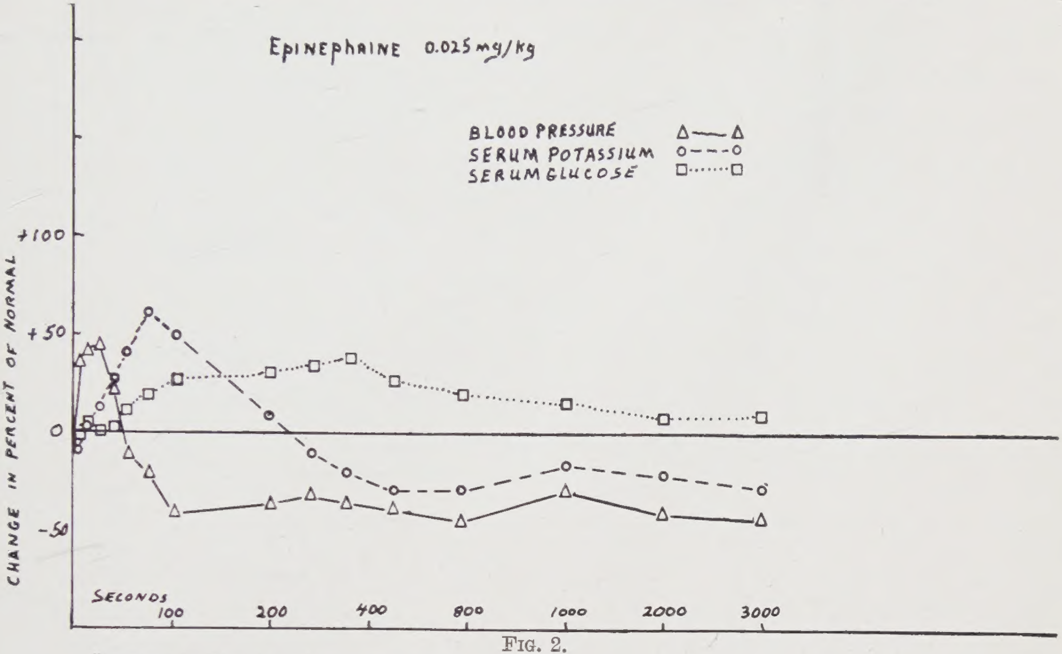

FIG. 2.
Changes in blood pressure, serum potassium and serum glucose following the intravenous injection of 0.025 mg/kg of epinephrine in a cat.

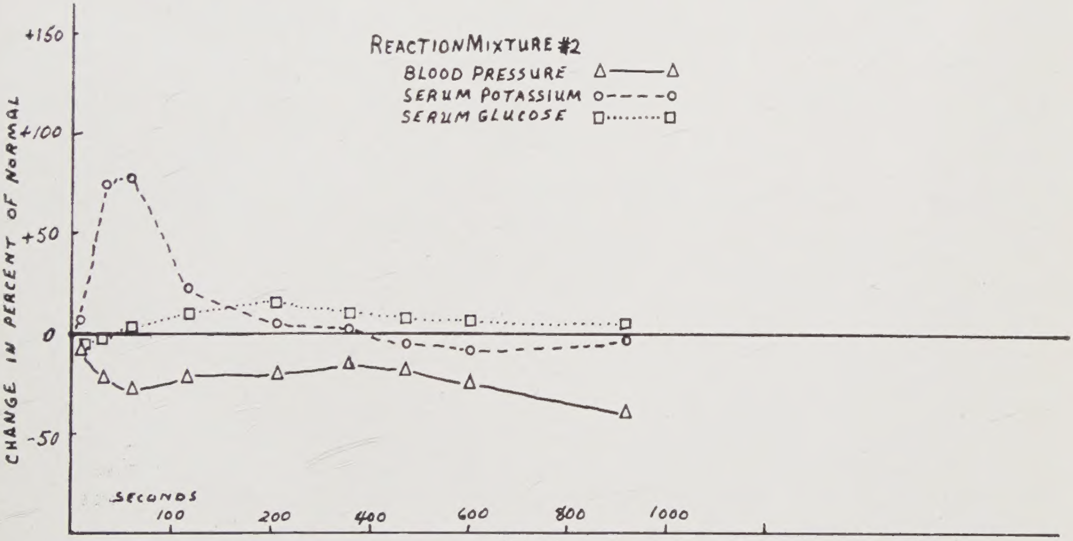

FIG. 3.
Changes in blood pressure, serum potassium and serum glucose following the intravenous injection of an oxidation product of epinephrine in the cat.

resulting adrenochrome as a pure crystalline compound from the reaction mixture failed, as they have for others (Green and Richter)[8]; because on concentration the ruby red color, presumably due to adrenochrome, disappears leaving a brown substance, probably melanin.

The crude reaction mixtures used in these experiments were prepared in each case just before use. In the first cat a reaction mixture No. 1 (which resulted from adding potassium permanganate calculated to supply 2 atoms of oxygen for each epinephrine molecule) in a dose of 5 mg per kg of original epinephrine resulted in a very marked diminution of the serum glucose rise, so that practically no change occurred. On the contrary the increases in blood pressure and serum potassium were comparable with those shown in Fig. 1. In consideration of the large dose, however, these increases were slight since greater effects are obtained even with 0.1 mg per kg of epinephrine.[5] Two samples of reaction mixture No. 2 were prepared in which potassium permanganate was added to supply 2½ atoms of oxygen per molecule of epinephrine. Each was tested on a cat. The serum glucose was little affected as with reaction mixture No. 1. The blood pressure however showed a fall instead of a rise in both animals. In one animal the serum potassium rose (Fig. 3) as it did with reaction mixture No. 1 but in the other it showed no change. These experiments although few in number gain significance by the fact that the regularity and degree of blood pressure and serum potassium changes produced by epinephrine have been previously established,[5] the same being true for the serum glucose changes as reported in the first part of this paper. Consequently any deviation from this constant effect is striking. These results suggest an independence of the three effects since in the oxidation of epinephrine they are not modified in parallel. The elimination of the serum glucose effect even before those of blood pressure and serum potassium would not support the postulation made above that this action is due to a decomposition product of epinephrine.

Summary. In a series of experiments on cats the changes in serum glucose, serum potassium and blood pressure produced by epinephrine were compared chronologically. The rise in serum potassium occurs later than the rise in blood pressure while the rise in serum glucose begins still later and is much more prolonged than the other 2 effects. This indicates that epinephrine hyperglycemia is independent of the serum potassium increase. As further evidence it is shown that in the oxidation of epinephrine by potassium permanganate the above actions are not modified in parallel.

14958

Tumor of Rat Testis Produced by Heterotransplantation of Infantile Testis to Spleen of Adult Castrate.

MORTON S. BISKIND* AND GERSON R. BISKIND.

From the Endocrine Laboratory, Beth Israel Hospital, New York, and the Departments of Pathology, Mount Zion Hospital and the University of California Medical School, San Francisco.

In a previous report,[1] we have described the development of granulosa cell tumors of the rat ovary after transplantation of one ovary to the spleen and removal of the other. With this technic, the estrogen produced by the transplanted ovary is secreted into the portal circulation and inactivated in the liver. The estrogen is thus prevented from exerting its normal cyclic inhibiting effect on the pituitary. The excessive secretion of pituitary gonadotrophin which thus results is enabled to act continuously on the transplanted gonad.

The present communication is concerned with application of this technic to the production of neoplasm of the rat testis. Implantation of an entire adult testis into the spleen of the same rat is impossible owing to the comparatively large size of the former organ; only small amounts of testicular tissue can thus be implanted. However, heterotransplantation of the testis of an infantile rat, from 9 to 14 days old, into the adult spleen, is comparatively as simple as similar ovarian

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* Aided by grants from the Lederle Laboratories, Inc., the Winthrop Chemical Company, Inc., and the United Hospital Fund of New York.

[1] Biskind, M. S., and Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 176.

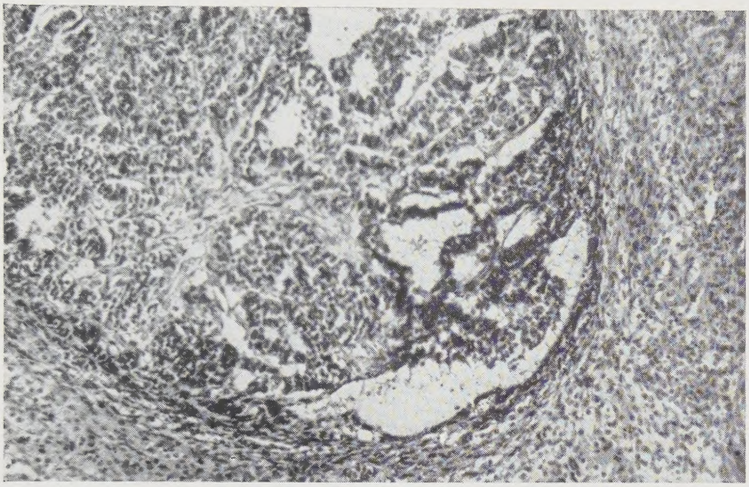

FIG. 1.
Nest of "granulosa" cells in interstitial cell mass. (About 90 ×.)

transplantation.

Accordingly, adult rats of the Sherman strain were castrated and a testis from an infantile rat of the same strain was implanted into the spleen of each. Similar implantation was also made into the spleens of noncastrate animals. The animals were killed at intervals of from 8 to 11 months after the implantation.

In 2 animals that had not been castrated, the implants had increased from an original size of about 5x2x2.5 mm to 8x5x5 mm and 11x5x5 mm in 8 months. In both cases, there was extensive degeneration of the tubules and little remaining interstitial tissue on microscopic examination. There were numerous large cystic areas.

In animals that had been castrated at the time of implantation, the picture was different. In one animal the transplant had increased in size from about 4x1.5x2 mm to 11x9x6 mm in 9 months. In another, the transplant grew in size from 5x2x2.5 mm to form a tumor 21x12x12 mm in size in 11 months; the latter was the largest of all the implants in this series (and exceeded the size of a normal adult rat testis); it formed an irregular, nodular mass which protruded at intervals from the surface of the spleen. In every case, in the animals that had been castrated before the transplantation was made, the prostate and seminal vesicles were atrophic, grossly and histologically, and the hypophysis showed the typical castrate change, indicating that no significant portion of the androgen secreted by the implanted gonad, escaped inactivation in the liver.[2]

Histologically, the large tumor is distinctly demarcated from the splenic substance by a laminated band of fibrous tissue that varies in thickness. Some strands of fibrous tissue arise from this capsule and radiate for variable distances into the tumor. Two distinct cellular components are readily recognized (Fig. 1 and 2), for the most part sharply demarcated from each other but in a few places intermingled. The major component is composed of a solid sheet of relatively large polyhedral cells that are compactly arranged and poorly outlined. They have a granular eosinophilic cytoplasm that may contain an irregular vacuole. The nucleus is round and contains many fine chromatin granules and a small basophilic nucleolus. An occasional mitotic figure is observed. A moderate amount of fibrous stroma permeates between the cells; the vascular tree is not readily evident. The second and more interesting component is made up of nests of small cells that have scant cytoplasm that may be vacuolated, a small nucleus containing many small chromatin granules and, rarely, a small basophilic

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[2] Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

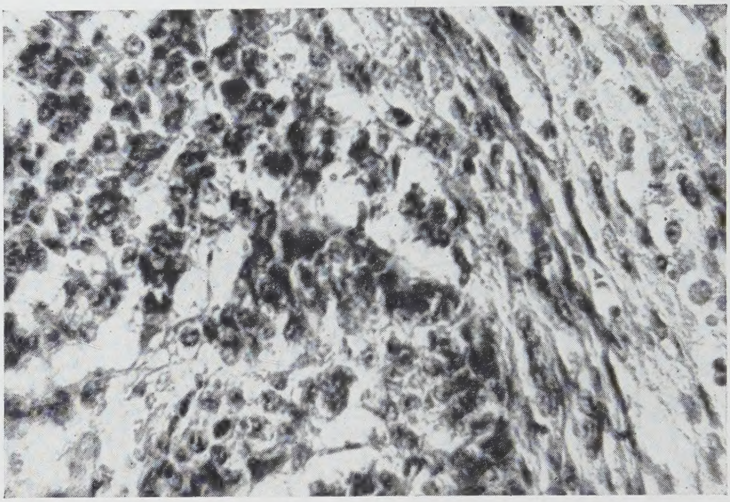

FIG. 2.
"Granulosa" cells and adjacent interstitial cells (about 240 ×).

nucleolus. Mitotic figures are fairly numerous. These nests often have a limiting fibrous strand and the cells are retracted from the strand in many places leaving irregular vacuoles. These small cells may be arranged in compact masses that sometimes take on a poorly developed adenomatous or papillary pattern. Small cysts are formed that may contain a pink staining coagulum. In several places there is an intermingling of the large cells and the nests of small cells. In addition to this intermingling, in many places there are minute nests of small cells entirely surrounded by the large cells. These minute collections are slightly larger than those of the spermatic tubules but are highly suggestive of a tubular origin, and are reminiscent of the pattern seen in arrhenoblastoma (Fig. 3). At one point there are small remnants of epididymis and some atrophic spermatic tubules. The splenic substance is not altered

Another implant, observed after 11 months, except for evidences of old hemorrhage around the fibrous capsule of the tumor.
is made up of fairly well preserved epididymal tubules and many atrophic spermatic tubules lying in a mass of large cells that must be derived from the interstitial cells. There is a slight increase in fibrous stroma between these cells, and in one place there is a deposit of basophilic granular material, probably calcium. The spermatic tubules are shrunken and empty except for a single layer of small vacuolated cells that have a pyknotic nucleus; this appearance is quite characteristic of the atrophy that occurs in maldescent of the testis.

A third implant, 11 months after insertion in the spleen, has a histologic structure quite similar to that of the mass just described, except that there is a greater proliferation of the interstitial elements and fewer tubules. In some regions the interstitial cells have a distinct granular cytoplasm; in other places most of the cells are vacuolated. There are many more vascular channels in this interstitial cell mass.

A fourth implant observed at 9 months is also of interest. The tubules have not undergone as extensive atrophy; some are lined by 1, 2, or 3 layers of cells. The row of cells that rests on the basement membrane is made up of larger cells, with vacuolated cytoplasm and somewhat vesicular nucleus. The upper rows are composed of smaller cells, poorly defined, that have a small round nucleus in which the chromatin is often placed in threads; these are remnants of the spermatogenic elements. In some of the degenerating tubules these cells appear piled up in one segment of the tubule, in which the basement membrane has disappeared, suggesting beginning invasion of the interstitial cell mass by tubular cells.

One of the most striking features of the

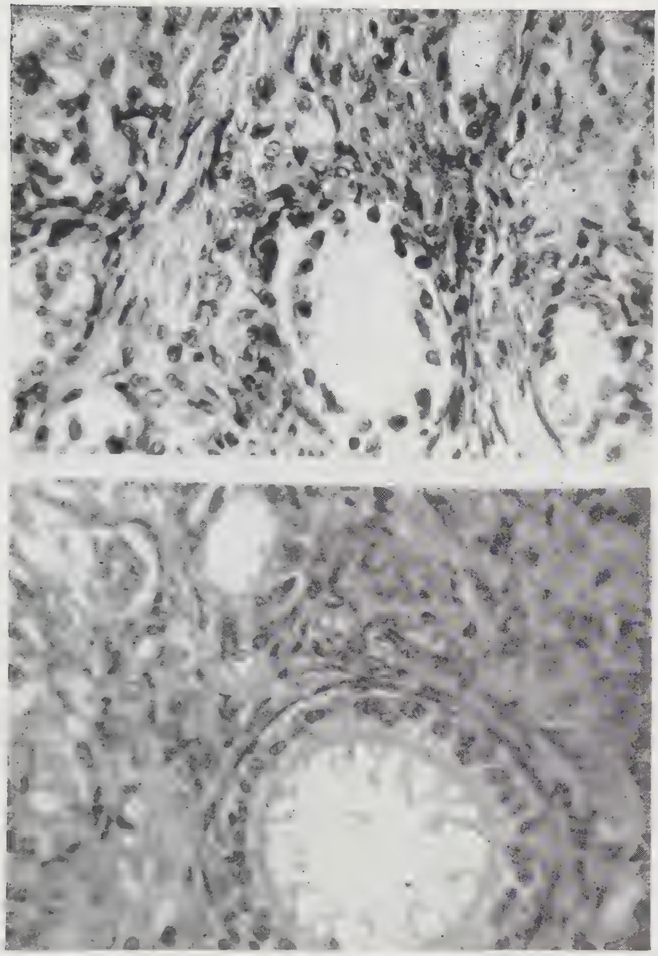

FIG. 3.
Arrhenoblastoma-like structures in interstitial cell mass (about 240 ×).

large tumor described (Fig. 1 to 3) is the resemblance of each of the two components, the small cells lying in irregular nests and the large cells arranged in sheets, to the two components observed in the tumors of the ovary produced by this method.[1] Not only is there a striking similarity of the small cells of the testicular tumor to the granulosa cells of the ovarian tumor, but even the interstitial cells of the testicular tumor closely resemble the theca elements of the other. In fact, were it not for the epididymal remnants, these two different tumors might be indistinguishable.[†]

Summary. The fact that estrogens and androgens are inactivated in the liver, served as the basis for studies on the effect of transplantation of the gonads to the spleen, which is in the portal circulation. Hepatic destruction of the gonadal steroids removes their normal inhibiting influence on the pituitary, permitting excessive secretion of gonadotrophin.

Implantation of the testis of an infantile rat into the spleen of an adult male castrate, resulted after 11 months in the development of a large tumor, bearing a striking resemblance to the granulosa cell tumor of the ovary produced by a similar method, previously reported. The cellular component apparently derived from the tubules and the interstitial cell mass in the testicular tumor, are indistinguishable from the granulosa cell and theca cell components, respectively, of the ovarian tumor. In addition, certain portions of the testicular tumor are reminiscent of the structure seen in arrhenoblastoma.

[†] We are indebted to Dr. Alfred Plaut and his staff for preparation of the histologic sections of the testicular implants.

14959 P

The Cause of Epidemic Diarrhea, Nausea and Vomiting. (Viral Dysentery?)

HOBART A. REIMANN, ALISON HOWE PRICE, AND JOHN H. HODGES.
From the Jefferson Medical College and Hospital, Philadelphia, Pa.

Tests were made to discover if the disease called epidemic nausea, vomiting and diarrhea[1] or infectious gastroenteritis is (a) caused by a filtrable agent and (b) transmitted as an airborne disease; (c) if the portal of entry is the respiratory tract or the gastrointestinal tract and (d) whether the causative agent is present in the oropharynx, intestine, or blood.

The experiments were made on medical student volunteers under conditions not best suited to work of this kind. The students for obvious reasons could not be segregated, the number of test subjects was limited, and since the material obtained from patients with the disease was immediately filtered and given to volunteers, the experiments were made during a declining natural epidemic. The tests were begun about 3 weeks after the peak of the epidemic[1] had passed and while only 1 or 2 remnant attacks occurred daily or at intervals of days.

Broth garglings and suspensions of diarrheal stool samples were filtered through medium Mandler filters, nebulized and applied to volunteers usually within a period of 2 to 4 hours after their collection. Bacteria-free filtrates were nebulized in Wells flasks[2] with compressed air, and could be seen by transmitted light emerging from the flasks as a fine, almost imperceptible mist. The stream was directed into a large pasteboard box into which the volunteer's head was placed for 5 minutes. Volunteers and the rest of the student body were then questioned at short intervals for 3 weeks for the development of symptoms.

Thirty-two volunteers in sets of about 8 on different days inhaled filtrates of garglings; of these 17 had attacks from 1 to 21 days afterward, mostly between 1 and 4 days. The symptoms reported were malaise, headache, abdominal discomfort, nausea, vomiting and diarrhea in varying combinations and in varying severity, mostly mild. In 7, mild nasopharyngitis occurred in addition.

Twenty-one volunteers inhaled stool filtrates and, of these, 11 developed similar symptoms in a similar period, 3 with mild nasopharyngitis also. No symptoms developed in 6 who inhaled nebulized serum.

Fifteen volunteers swallowed 3 cc of filtered garglings enclosed in double gelatin capsules, 5 swallowed stool filtrates, and 4 swallowed serum each similarly encapsulated. No significant symptoms developed in any.

In the remainder of the 240 students in the classes from which the volunteers were drawn, 22 attacks apparently of the declining epidemic disease occurred during the 4-week period while the experiments were in progress, as shown in Fig. 1. Some of these may have been contracted from ambulatory, sick volunteers.

Summary. Among the 32 volunteers who inhaled nebulized filtered garglings, symptoms developed in 17; of the 21 who inhaled nebu-

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[1] Reimann, H. A., Hodges, J. H., and Price, A. H., *J. A. M. A.*, 1945, **127**, 1; Reimann, H. A., Price, A. H., and Hodges, J. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 233.

[2] Wells, W. F., to be published.

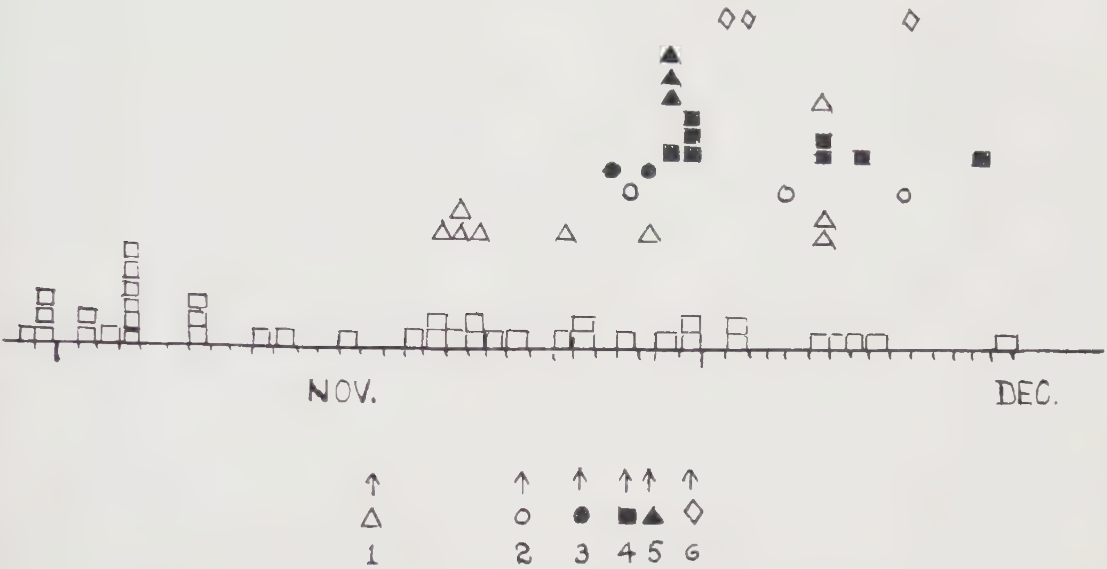

Fig. 1.

The natural epidemic among students compared with the number of induced attacks. The hollow squares represent natural attacks among 240 students in a declining epidemic; 22 attacks occurred after November 15 when the first experiment began. In the 6 experiments, 28 attacks among 53 volunteers are charted in various symbols. The 6 arrows and symbols indicate the date of exposure of volunteers to filtrates, and the corresponding symbols above indicate the day on which the respective attacks occurred.

lized stool filtrates, 11 developed symptoms. Of the combined groups of 53 volunteers, 28 (53%) developed symptoms as compared with 22 out of 240 (9%) of the rest of the student body in which the natural disease apparently occurred during the period of the tests. No attacks occurred in 6 volunteers who inhaled serum nor in 24 volunteers who were fed serum or filtrates of garglings or stools.

The results, considering the unideal circumstances during which the tests had to be made, suggest that the causative agent of the disease is filtrable, airborne, enters through the respiratory tract and is present in the oropharynx and stool but not in the blood. Tests are now being made in a nonepidemic period with material from the November epidemic preserved at –70°C.

14960

Methods for Determination of Thiamine in Blood and Tissues with Observations on Relative Contents.

Louis D. Greenberg and James F. Rinehart.

From the Divisions of Pathology and Pharmacology, University of California Medical School, San Francisco.*

A number of methods have been proposed for the determination of thiamine in blood. Among these are the Phycomyces growth test described by Rowlands and Wilkinson,[1] and by Meiklejohn,[2] the cocarboxylase method of

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* Aided by a grant from the Christine Breon Fund for Medical Research.

[1] Rowlands, E. N., and Wilkinson, J. F., *Brit. Med. J.*, 1938, **2**, 878.

[2] Meiklejohn, A. P., *Biochem. J.*, 1937, **31**, 1441.

Goodhart and Sinclair,[3] and the yeast fermentation method of Schultz, Atkin and Frey.[4] Thiochrome methods have been proposed by a number of authors, namely, Ritsert,[5] Hennessey and Cerecedo,[6] Hennessey and Lewis[7] and also Friedemann and Kmieciak.[8] The procedure of the last-named authors offers considerable simplification over the previous thiochrome methods.

We have been using a method, during the past 2 years, for the determination of thiamine in blood, which we believe represents an even more simplified thiochrome procedure than any heretofore proposed. In addition the authors' method has the advantage that it can be carried out on as little as 1 cc of blood. It yields results which are in agreement with those found by Goodhart and Sinclair,[3] with the cocarboxylase assay; with those of Goodhart and Nitzberg[9] using the fermentation method, with those of Friedemann and Kmiechiak,[8] and also with those of Benson *et al.*[10] using thiochrome procedures. The values obtained on a group of "normal" individuals (laboratory associates and medical students) with the present method range from 5.0-10 μg per 100 cc.

Our experience, in agreement with others, has been that the total thiamine of the blood of humans is relatively static or immobile and hence is not affected rapidly by the intake of thiamine. This is particularly true when the levels are above 5 μg per 100 cc.

Actually, in order to convincingly demonstrate that blood thiamine determinations are of value in the assessment of deficiency states, it would be worth while knowing if there is good correlation between the blood thiamine and tissue concentrations of man and animals. We have investigated this problem in the rat and the data is presented in this publication. At this point it appears to be timely to make some remarks concerning differences which we have observed between rat bloods and human bloods. In the first place, on an adequate intake the concentration of thiamine in rats' blood is usually somewhat higher than that found for humans; secondly, the blood of the rat appears to reflect more sensitively the changes in the dietary intake of thiamine than does the blood of man.

Method. To 10.5 cc of H_2O in a test tube is added 3.0 cc of oxalated blood, followed by the addition of 1.5 cc of 5% metaphosphoric acid. The mixture is stirred well and heated for 10-15 minutes on a boiling water bath with occasional stirring. It is cooled, 6 cc of water and 4 cc of acetate buffer, pH 4.5, are added with stirring until the whole coagulum is in a fine state of suspension. Following centrifugation the clear supernatant fluid is decanted into another test tube, 200 mg of takadiastase (Parke-Davis) is stirred into the clear liquid and a drop of toluene is introduced. The test tube is stoppered and the extract is incubated over night at 45°-50°C. Following incubation the digest is recentrifuged and 10 cc of the supernatant fluid is adsorbed on Decalso in the manner described below. With 2 cc of whole blood proportionate quantities of water and reagents are employed. The rest of the procedure is the same.

Preparation of the Filter. 500 mg of Decalso, washed and prepared according to the method of Hennessey and Cerecedo,[6] is introduced into a pyrex filter tube (30 cc cap.) with sintered glass filter of medium porosity containing 8-10 cc of 3% acetic acid. The filter tube is placed in a suction filter flask or bottle. Gentle suction is applied by means of a water pump and the acetic acid is drawn through the filter.

Adsorption and Elution of the Thiamine. 10 cc of the takadiastase-digested extract is filtered through the filter slowly (at least 10 minutes being allowed for its passage). The adsorbent is washed with 3 successive 10 cc portions of water and then sucked dry. The stem of the filter tube is wiped dry and a small test tube is placed under it for the collection of the eluate. The adsorbate is stirred up with 2 cc of 25% KCl in 0.1 N HCl and allowed to settle. After standing approximately 5 minutes or longer, gentle suction is applied and the KCl is sucked through the filter. When all of the first portion of the KCl solution has passed through, a second 2 cc portion is drawn through the filter followed by an additional 1 cc portion of the KCl solution. The filter is then sucked dry and the collection tube is removed from the suction bottle.

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Oxidation of Thiamine. For the oxidation of thiamine, the procedure is similar to that of Harris and Wang.[11]

Two cc portions of the KCl eluate are transferred to Babcock cream test bottles. Two cc of methyl alcohol, 0.3 cc of 1% potassium ferricyanide solution, 1 cc of 30% NaOH and 10 cc of isobutyl alcohol are added in this order and the bottles are shaken vigorously for 1.5 minutes. A drop of 3% H_2O_2 is now added to destroy any excess of ferricyanide remaining in the vessels after the oxidation period. In agreement with the work of Ellinger and Holder[12] we have observed that ferricyanide causes a quenching of the thiochrome fluorescence. The blank is treated in the same way as the unknown with the exception that the ferricyanide is omitted. The mixtures are centrifuged slowly for 1 minute and 8 cc portions of the isobutyl alcohol extracts are pipetted into fluorometer tubes.

Readings of the isobutanol extracts are made with the Coleman photofluorometer model 12, standardized each time against pure thiamine samples oxidized in the same manner as the unknown. Coleman light filters (B_1 or B_1-s as the primary, and PC as the secondary) have been employed in the instrument while making fluorescence measurements.

Tissue Thiamine Methods. 0.3 to 1 g quantities of tissue are ground in a mortar with sand and distilled water to a fine mince and transferred to a test tube with water until a total of 13.5 cc of water has been used for grinding the tissue and for washing the mortar. The tissue suspension is then placed in a boiling water bath and after 1-2 minutes, 1.5 cc of 5% metaphosphoric acid is added and the suspension is mixed well. Heating is continued for 20-25 minutes with occasional stirring. The samples are cooled and centrifuged. The supernatant fluid is decanted into 25 cc volumetric flasks and the residue washed twice by centrifugation with 2 successive 5 cc portions of 0.5% metaphosphoric acid. The washings are added to the extract and the volume made up to 25 cc with distilled water.

A 5 cc portion of the tissue extract is mixed with an equal amount of acetate buffer pH 4.5 and incubated over night with 0.1 g of takadiastase. Although a 4-hour incubation period has yielded substantially the same results as over-night incubation, we have preferred the latter because of its greater convenience. Following incubation the thiamine in the whole of the takadiastase-treated sample is adsorbed, eluted and oxidized to thiochrome in the manner described for the analysis of blood thiamine. In our hands this method has yielded values which are in agreement in most instances with those reported by others.[13-16]

Experimental. 12 rats weighing 130-166 g were divided into 2 groups of 6 animals each. One group received a vitamin B_1-deficient diet, and the other the same diet supplemented with thiamine. The basal diet consisted of sucrose 68%, casein 18%, Crisco 8%, salt mixture (Hawk and Oser) 4%, and cod liver oil 2%.

The ration was supplemented by pipette daily with 2 drops (approximately 0.1 cc) of a vitamin mixture of the following composition: thiamine chloride 0.08 g; riboflavin 0.08 g; pyridoxine hydrochloride 0.08 g; nicotinic

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TABLE I.
Blood and Tissue Thiamine of Thiamine-Deficient and Control Rats.

Rat Nos.	Deficient or control	Days on diet	Blood, μg per 100 cc	Skeletal muscle, μg per g	Heart ventricle, μg per g	Kidney, μg per g	Liver, μg per g
2, 6	control	17	10.9	0.7	4.9	4.3	5.6
1, 3	deficient	17	3.9	0.3	2.6	1.6	3.2
1_c, 4	control	45	13.1	0.8	3.7	—	4.1
4, 5	deficient	45	1.5	0.2	0.5	—	0.5

TABLE II.
Blood and Tissue Thiamine.

Rat				Thiamine				
Group	No.	Days on diet	Thiamine dosage, μg per day	Blood, μg per 100 cc	Muscle, μg per g	Heart ventricle, μg per g	Kidney, μg per g	Liver, μg per g
I	6	26	20	8.3	0.7	3.1	1.7	3.0
I	4	33	20	7.5	0.6	2.0	1.0	1.8
I	16	40	20	7.4	0.7	3.8	1.8	3.5
II	20	26	40	20.4	1.8	6.8	6.3	8.6
II	4	33	40	17.7	1.4	4.0	4.0	7.5
II	12	40	40	14.6	1.1	4.8	3.1	4.7

acid 1.0 g; choline chloride 10 g; calcium pentothenate 0.1 g; and para-aminobenzoic acid 0.15 g, dissolved in 100 cc 20% ethyl alcohol. The thiamine chloride was omitted from the supplement used for the animals on the deficient regime.

After a period of 17 days and again after 45 days on the diet 2 control and 2 deficient animals from which food and vitamin supplements had been withheld for 18-20 hours, were sacrificed on each occasion. This was carried out by anesthetization with ether and severance of the large vessels leading to the heart following opening of the thoracic cavity. The lungs were removed and the blood permitted to flow into the pleural cavity from which it was aspirated with a syringe and introduced into a test tube containing sodium oxalate. Heart, kidneys, liver and portions of skeletal muscle from the hind legs were removed and equal portions of each respective tissue from each of two animals were pooled for thiamine analysis. Blood thiamine analysis was also carried out on pooled samples.

The results of analysis of tissues and blood from animals suffering depletion of thiamine for 17 days and 45 days, along with the values for tissues of control animals are summarized in Table I. The data show that the blood thiamine level of the rat parallels the tissue concentration of the vitamin during periods of depletion.

Some additional data taken from a current, paired feeding experiment on riboflavin deficiency, in which only the data on control rats is used, demonstrates clearly the parallelism between the blood and tissue concentrations of thiamine in the rat. Blood and tissues rather sharply reflect the vitamin B_1 intake. The basal diet and supplements were the same as described earlier in this publication with the exception that each of 2 groups of control rats received 20 and 40 μg of thiamine respectively. The animals weighed 56-78 g when they were placed on the diet and were sacrificed after 26-40 days. Tissues and blood of individual animals were analyzed for thiamine by the procedures outlined in this report. In this instance 1 cc quantities of blood were subjected to analysis and proportionate amounts of reagents were used throughout with the exception that the thiamine was eluted with a total volume of 5 cc of 25% KCl in 0.1 N HCl. Two cc portions of the eluate were used for the oxidation. The results are summarized in Table II. Since these animals were restricted to their food intake, there is present a factor of partial starvation. While this might increase somewhat the thiamine values in each group, the relationship under consideration would not be affected.

Summary. With the aid of these methods it has been shown, in the rat, that the thiamine concentration in the blood parallels that in the tissues, and both reflect the intake.

14961

Depression of Acetycholine Synthesis by Serum from Working Muscle. Healthy Subjects and Myasthenia Gravis Patients.*

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In the presence of serum from patients with myasthenia gravis, a persistent state of easy fatiguability,[1,2] less acetylcholine is synthesized than in the presence of serum from healthy subjects. The purpose of the following study was to investigate the effect of exercise in both healthy subjects and patients with myasthenia gravis measuring the action of serum on acetylcholine synthesis. The results are of particular interest with respect to the mechanism of the genesis of fatigue.

Method. Experiments were performed on 12 healthy subjects and 5 patients with myasthenia gravis. The subject alternately flexed his fingers, forming a fist, and extended them fully, performing the entire action rhythmically, at the rate of one contraction per second. A sphygmomanometer cuff was applied to the arm and inflated during the fourth to ninth contraction of the fingers to a pressure of 250 mm Hg. Ten cc blood was collected from the brachial vein. Blood collected from the other immobilized arm with occluded circulation served as control. The blood was centrifuged immediately after collection, oxygenated thoroughly, and the effect of serum on the synthesis of acetylcholine was tested by the method of Quastel, Tennenbaum, and Wheatley[3] with minor modifications.[2] One hundred mg fresh minced frog brain, 1 cc serum, 2 cc Ringer's solution of pH 7.4, 4.8 mg glucose, and 3 mg physostigmine salicylate were incubated and shaken aerobically for 4 hours at 37°C. The amount of acetylcholine

TABLE I.
Effect of Serum Collected During Muscle Contraction on Synthesis of Acetylcholine.

Subject	No. of first formation until fatigue	Amt acetylcholine synthesized by serum from resting arm in % of healthy control serum*	Amt acetylcholine synthesized with serum from working arm in % of own control	Total decrease of acetylcholine synthesized
Twelve healthy subjects	60-105	100	60 ± 1.6	40
Patients with myasthenia gravis:				
R	15	64	85	45
L	30	72	80	43
S	50	76	74	44
V	20	74	83	39
M	40	78	76	41

* The amount of acetylcholine synthesized in the presence of serum from the control arms of healthy subjects averaged 2.08 μ.

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* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

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TABLE II.

Short Summary of the Clinical State of the 5 Patients with Myasthenia Gravis.

Name	Sex	Age	Severity of disease	Duration (years)	Thymectomy	X-ray treatment	Symptomatology	Prostigmine Bromide (Hoffmann-La Roche) mg per day: Amt before thymectomy	Prostigmine Bromide (Hoffmann-La Roche) mg per day: Present amt	Achievement after medication
R	F	24	3+	10	no	yes	Moderate lid ptosis, occasional diplopia and difficulty in chewing, moderate muscular fatiguability		90	Walks 1-2 blocks
L	M	46	3+	1	,,	no	Severe lid ptosis, constant diplopia, mod. musc. fatig.		60	Walks
S	M	23	2+	3	yes	yes	Slight lid ptosis, rare diplopia, mod. musc. fatig.	120	15 to 30	Works as engineer
V	F	21	2+	6½	no	no	Slight lid ptosis, rare diplopia, occasional difficulty in chewing, mild musc. fatig.		45	Walks, goes to parties
M	F	28	2+	7	,,	,,	Slight lid ptosis, rare diplopia, occasional difficulty in chewing, mild musc. fatig.		45	Works as cashier

synthesized was assayed biologically with the sensitized rectus abdominis muscle of the frog. The amounts of acetylcholine synthesized in the presence of serum obtained from the working arm was expressed as percent of the control.

Results. The amounts of acetylcholine synthesized are given in Table I and Fig. 1. Frog brain incubated without serum synthesized on the average 1.02±0.033 μg acetylcholine per 100 mg of frog brain; mixtures of frog brain and serum from immobilized arms of healthy subjects synthesized 2.08±0.080 μg acetylcholine.[2] The amount of acetylcholine synthesized in the presence of serum from healthy subjects deviated from the average less than ±8%.

Healthy subjects contracted the fingers from 75 to 105 times before severe fatigue appeared. Approximately 40% less acetylcholine was synthesized in the presence of serum collected after performance of the contractions by the healthy subjects than in the presence of the control serum.

A short summary of the clinical state of the patients with myasthenia gravis is given in Table II. Acetylcholine synthesis in the presence of serum from the immobilized arm of patients with myasthenia gravis was less than in the presence of serum from the immobilized arm of healthy subjects. The more severe the myasthenia gravis the less the acetylcholine synthesis. The patients contracted the fingers from 15 to 50 times before severe fatigue appeared. Serum collected from patients with myasthenia gravis after performance of the contractions decreased the synthesis by 15 to 26% as compared to the serum collected from the resting arm of the same patient. Since the serum obtained from the resting arm of these patients depressed the synthesis of acetylcholine by 22 to 36% as compared to the effect of serum from the resting arm of healthy subjects, the total decrease of the acetylcholine synthesis in the patients with myasthenia gravis after performance of muscle work averaged 42%. These results suggest that when the blood changes during performance of muscle contractions as to decrease the synthesis of acetylcholine *in vitro* by 40%, performance of

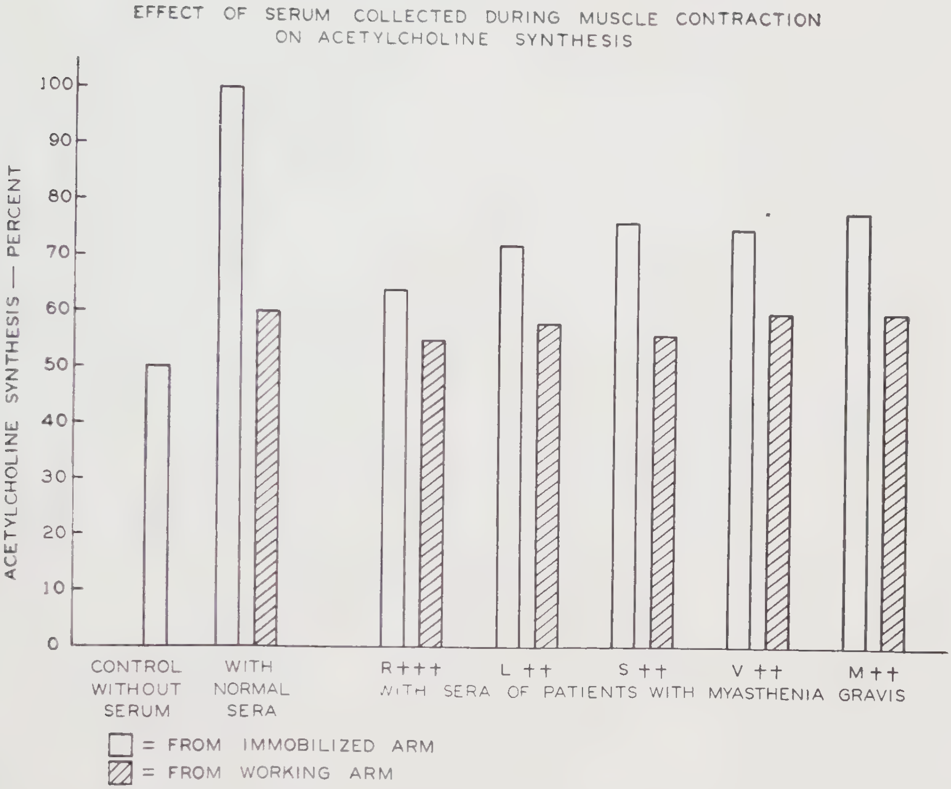

FIG. 1.
Effect of Serum Collected During Muscle Contraction on Acetylcholine Synthesis.
The average amounts of acetylcholine synthesized are expressed as percent of the amount synthesized in the presence of serum from the immobilized arm of healthy subjects.

voluntary muscle work becomes exceedingly difficult.

Discussion. The above experiments suggest that during performance of muscle work with occluded circulation changes of the physico-chemical state at the neuro-muscular junction may result in a decrease of the local synthesis of acetylcholine. It seems that voluntary muscle contraction becomes extremely difficult when the agents involved reduce the acetylcholine synthesis *in vitro* to a similar extent in both healthy subjects and patients with myasthenia gravis.

Should the biochemical changes responsible for the above results also occur during performance of work by muscle with adequate blood supply, muscle fatigue may at least partly be the result of the decreased local synthesis of the acetylcholine-like substance.

It is known that during prolonged activity of cholinergic systems an acetylcholine-like substance is synthesized locally.[4,5] A local synthesis of acetylcholine is conceivable since acetylphosphate,[6] a possible intermediate product of the anaerobic carbohydrate and fat metabolism, may be one of the precursors of acetylcholine. Among others many intermediate products of carbohydrate metabolism[7,8] and many amino acids[9,10] increase the synthesis of acetylcholine (as tested by the modified method of Quastel, Tennenbaum, and

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[4] Brown, G. L., and Feldberg, W., *J. Physiol.*, 1936, **88**, 265.

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[10] Torda, C., and Wolff, H. G., in press.

Wheatley[2]). On the other hand decomposition products of adenosinetriphosphate[8,11] decrease it. The local concentrations of inorganic ions may also exert some effect on the synthesis.[3] Our results with serum from working muscle suggest that during prolonged muscle work the dynamic equilibrium may be shifted to cause decrease of the local acetylcholine synthesis. If such event does occur it may contribute to the genesis of muscle fatigue.

At present it cannot be stated whether the decrease of acetylcholine synthesis was due to a depressor substance released from the muscle during contractions or whether it was due to utilization of some potentiator substances ordinarily present in the blood and used by the muscle to perform work. The existence of potentiator substances in the blood can be assumed since the amount of acetylcholine synthesized *in vitro* in the presence of serum from the immobilized arm of healthy subjects is twice as much as the amount synthesized in the absence of serum.

The implications of these results regarding myasthenia gravis are: 1. In the presence of serum[1,2] from patients with myasthenia gravis less acetylcholine is synthesized than in the presence of serum from resting healthy subjects. The factor responsible for these effects could be (I) lack of the usually occurring potentiator constituents of the serum; (II) a depressor agent being: either (a) a foreign substance;[12] or (b) an intermediate product of body metabolism;[11,13,14,15] or (c) a substance released during muscle contraction but not adequately metabolized. (2) In patients with myasthenia gravis the depressor effect of blood that has circulated through the muscle during contraction adds to the effect of blood circulating before voluntary muscle contractions. Muscle fatigue appears as soon as the total decrease is approximately that occurring in healthy subjects at the onset of fatigue.

Summary. 1. The effect of muscle work of healthy subjects and patients with myasthenia gravis on the ability of serum to modify acetylcholine synthesis *in vitro* was investigated. 2. Acetylcholine synthesis in mixtures of frog brain and serum from immobilized arm of healthy subjects was twice as much as in the absence of serum. 3. Acetylcholine synthesis in the presence of serum from working arm was 40% less than in the presence of serum from immobilized arm. 4. Acetylcholine synthesis in the presence of serum from the immobilized arm of patients with myasthenia gravis was less than in the presence of serum from the immobilized arm of healthy subjects. The more severe the myasthenia gravis, the less the acetylcholine synthesis. 5. Acetylcholine synthesis in the presence of serum from the working arm of patients with myasthenia gravis was 15 to 26% less than with serum of their own immobilized arm, but on the average 42% less than the amount synthesized in the presence of serum from the immobilized arm of healthy subjects. 6. Muscle fatigue may be in part the result of the decrease of the local synthesis of an acetylcholine-like substance during prolonged muscle contractions. 7. Implications regarding the symptomatology of myasthenia gravis are presented.

The authors wish to express their gratitude to Lieut. Jos. L. Lilienthal, Jr., (MC) USNR, for suggesting the problem.

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[15] Torda, C., and Wolff, H. G., in press.

14962

Calcium Content of Tissues from Rats Receiving Different Sulfonamides.*

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During studies[1] on the enzymatic changes in tissues from rats fed sulfasuxidine, a marked increase in the calcium content of the liver tissue was observed. Indirect evidence that concentrates from liver were effective in counteracting the change was also obtained. In this paper we wish to report a more detailed investigation of these changes and of the efficacy of liver concentrates in reversing the accumulation of excess calcium. Attempts have also been made to elucidate the mechanism of the action by studying the effect of other compounds which have pharmacological effects in common with those of some of the sulfonamides. Martin[2] found several sulfonamides, thiourea and *p*-aminobenzoic acid to produce a hyperplasia of the thyroid and hypothyroidism and to cause a "pseudo-potentiation" of the action of insulin. However, sulfadiazine, the sulfonamide showing the greatest anti-thyroid activity,[3] failed to produce changes in the calcium content of any of the tissues except the kidney, and this change may be attributed to other causes. *p*-Aminobenzoic acid and thiourea caused no change in the amount of calcium in any of the tissues analyzed.

Experimental. Animals. Twenty-one day-old male rats of the Sprague-Dawley strain weighing 35-45 g were placed on the following purified ration: casein (Labco, vitamin-free), 18; sucrose, 73; salts IV,[4] 4; corn oil, 5. The drugs were incorporated individually in place of part of the sucrose at the following percentages: succinylsulfathiazole, 0.75; sulfathiazole, 0.75; sulfadiazine, 0.50; *p*-aminobenzoic acid, 1.0; thiourea, 0.50. Each rat received the following B vitamins daily in 0.5 cc of 20% EtOH in a supplement dish: thiamin hydrochloride, 20 γ; pyridoxin hydrochloride, 25 γ; nicotinic acid, 25 γ; riboflavin, 30 γ; calcium pantothenate, 200 γ; choline hydrochloride, 10 mg; biotin (as SMA conc. No. 1000), 1 γ. Each rat received on alternate days 2 drops of corn oil containing the fat-soluble vitamins, in concentrations to allow the following *daily* intake: A, 30 I.U.; D, 30 I.U.; E (α-tocopherol), 0.4 mg; K (2-methyl-1,4-naphthoquinone), 20 γ. When solubilized liver (Wilson fraction "L") was fed as a therapeutic measure it was placed in the B-vitamin supplement at a level of 0.2 g per day.

The animals were maintained on these regimens for 2-12 weeks. Rats from each group were sacrificed at frequent intervals for calcium and histological studies, and also for correlation of calcium content with changes in enzymatic activity previously reported.[1] The rats were decapitated and drained of blood preparatory to removal of the tissues for the various studies.

Calcium Determination. Samples of liver, kidney, and leg muscle were blotted with moistened filter paper, weighed, dried at

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We are grateful to Merck and Co., Inc., for the B vitamins and α-tocopherol, to Wilson and Company for liver fraction "L," to Winthrop Chemical Co., Inc., for crystalline vitamin D, to Abbott Laboratories, for the haliver oil, and to Sharp and Dohme for the sulfonamides used in these experiments.

We are indebted to Dr. Milton D. Bosse of the Western Pennsylvania Hospital, Institute of Pathology, Pittsburgh, Pa., for the examination of the liver sections.

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TABLE I.
Calcium Content of Livers of Rats Fed Sulfonamides.

Weeks on exp.	Sulfasuxidine basal*	Sulfasuxidine plus Sol. liver*†	Sulfathiazole basal*	Sulfathiazole plus Sol. liver*†	Sulfadiazine basal*	Control, no drug*
2-4	39.5 43.0		7.5 12.5			
4-6	85.0 876 1480 1660		10.5 85.5 284 1287		10.5 11.0	20.5 23.0
6-8	15.0 88.5 191 577 944 1065 2380		9.5 9.5 23.5 785 1605		18.0 25.0 38.0	10.0 19.0 19.5 22.0 24.0
8-10	159 620 1230 1270 1280	12.0 63.5	791 1410	7.0 17.0	11.0 23.0	7.5 13.5 18.0
10-12	360 850 2640	14.5 14.5 23.5	24.0	7.5 24.0	10.0 11.5 14.5 16.0	9.5 12.5

* In milligrams percent of dry tissue.

† 0.2 g per day of solubilized liver was fed for 10-37 days after rats had been on basal sulfonamide ration at least 6 weeks and were continued on the sulfonamide during therapy.

105°C for 24 hours, and again weighed to determine the dry matter content. The dried tissue was ground and a 0.1-0.2 g sample ashed in a platinum crucible at 550-600°C. This procedure retains all of the calcium;[5] a slight residue of carbon does not interfere.[6] The ashed samples were treated essentially according to the method of Kuttner and Cohen.[7] They were dissolved in 0.2 ml of 2 N HCl, transferred with several small portions of water (total, 2 ml) to a special 15 ml centrifuge tube having a finely-drawn tip (an aliquot was taken for samples having a high calcium content), and precipitated with 0.5 ml of 1% Na_3PO_4 and 0.4 ml of 25% NaOH. The precipitate was washed twice with alkaline 50% ethanol and the phosphate determined in the Evelyn colorimeter by the method of Fiske and Subbarow.[8]

Histology. Within 15 minutes after the death of the animal, small portions of the liver were placed in Bouin's Fixative. After 24-48 hours they were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results. Values for the calcium content, in milligrams percent of dry tissue, of the individual livers of the rats on the various sulfonamide-containing diets are presented in Table I. With but one exception, all animals receiving the basal sulfasuxidine ration without liver exhibited a moderate to marked rise in calcium content within the experimental period of 2 to 12 weeks. Nevertheless, rats on this diet for 8 to 12 weeks, when fed 0.2 g per day of solubilized liver for 10 to 37 days, although continued on the sulfasuxidine ration, showed a return to normal values, except for one animal that was moderately above normal.

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TABLE II.
Dry Matter Content of Livers of Rats on Various Regimens.

Group	No. of samples	% dry wt*	Standard deviation†
Sulfasuxidine‡	8	27.85	1.21
,,	9	26.81	0.95
Sulfathiazole	7	28.20	0.57
Basal control (food *ad libitum*)	7	30.06	0.80
Control§ (restricted food intake)	8	30.75	0.56
Biotin deficient§	8	31.04	1.18

* The liver was rinsed, blotted, to remove excess fluid, and dried at 105°C for 24 hours.

† Calculated by the formula for a small number of samples:

$$\sigma = \sqrt{\frac{\Sigma d^2}{N - 1}}$$

‡ Animals from a previous experiment.[1]

§ Animals from other experiments[10,11] included for purposes of comparison.

Only one-half of the rats fed sulfathiazole had abnormal calcium levels, but these were as marked as those of the sulfasuxidine group. The calcium values of the livers of all animals in this group fed solubilized liver were normal. None of the livers of rats fed sulfadiazine, nor any of those fed thiourea or *p*-aminobenzoic acid showed any variations outside of the range of those of the normal control animals.

Histological examination showed no evidence of deposition of *inorganic* calcium salts. Several livers of the sulfasuxidine and sulfathiazole groups showed changes similar to those which have been reported.[9] There was moderate to severe swelling of a few to numerous liver cells both in central and portal areas. The cytoplasm of these cells was clear, suggesting hydropic changes or the presence of lipoid substances. Occasional foci of multinucleated liver cells and a few small areas of necrosis were noted in several of these sections. Some proliferation of Kupffer cells also occurred in some areas of a few sections. Occasional sections showed mild typical fatty change with small vacuoles in the cytoplasm of the liver cells of central zones. None of the sections of livers from the sulfadiazine group showed significant changes.

Livers containing large quantities of calcium were, on the average, approximately 50% larger than those in normal rats on the basis of total body weight. Enlargement of the livers of rats receiving sulfonamides has been reported by others.[9]

The livers of rats receiving the basal sulfasuxidine or sulfathiazole ration without liver also showed (by statistical analysis) a significant decrease in dry matter content. These data are presented in Table II, and values obtained from animals on other experiments[10,11] are included for comparison. Livers of animals supplemented with solubilized liver had a normal moisture content. Variations in moisture content of the livers from the sulfadiazine group do not permit proper evaluation of the results. These varied from 27-30% dry matter content.

In Table III are presented the calcium values for kidney and muscle tissues from the various groups of animals. Sulfadiazine produced (except for one value not included in the table) a definite increase in the calcium levels of the kidney. Sulfasuxidine and sulfathiazole caused a slight decrease in the calcium content of muscle. The calcium of tissues of rats fed *p*-aminobenzoic acid or thiourea was normal.

Discussion. Numerous reports in the literature indicate that sulfonamides exert toxic effects on animals and that they also decrease the synthesis of nutritional factors by bacteria in the gastro-intestinal tract (*cf.* [1]). Our data show that sulfasuxidine and sulfathiazole produce a marked accumulation of calcium in the liver and that this effect is reversed or prevented by feeding solubilized liver (Wilson

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[9] Gross, P., Axelrod, A. E., and Bosse, M. D., *Am. J. Med. Sci.*, 1944, **208**, 642.

[10] Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 237.

[11] Pilgrim, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **156**, 257.

TABLE III.
Calcium Content of Kidney and Muscle Tissues of Rats Receiving Sulfonamides.

	Kidney		Muscle	
Group	No. of samples	Calcium content*	No. of samples	Calcium content*
Sulfasuxidine	6	34.3 (18.5-68.0)	4	16.8† (11.0-23.5)
Sulfathiazole	5	31.1 (19.5-46.0)	5	19.3 (14.0-29.5)
Sulfadiazine	9	165.0 (99.0-336.)	4	33.1 (21.0-48.0)
Basal control	8	39.1 (19.5-54.0)	6	32.2 (20.5-49.5)

* In milligrams percent of dry tissue.
† One value of 183 mg% is omitted because the muscle appeared to be bruised.

fraction "L"). Only those livers having an increased calcium content exhibited a decreased enzymatic activity; the results previously reported[1] showing that feeding of "norite eluate" concentrates allowed normal enzymatic activity, are, therefore, an indirect indication that concentrates high in the "norit eluate factor," as assayed by *S. fecalis* (*S. lactis*) or *L. casei*, are effective in preventing this disturbance in calcium metabolism.

The alteration of the calcium metabolism can be attributed to: (a) a nutritional deficiency caused by a decreased rate of synthesis of factor(s) by the intestinal bacteria; (b) a toxicity of the sulfonamide which is evident only in the nutritionally-deficient animals; (c) a direct toxicity of the drug requiring a more or less specific antagonist. It seems most likely that the effect is the result of a nutritional deficiency, because: (a) sulfasuxidine is the least readily absorbed of the drugs tested, yet it produces the change most consistently; (b) the change can be prevented or reversed by feeding a concentrate known to be rich in nutritional factors.

The histological studies show that there are no deposits of inorganic calcium salts such as those that follow necrosis or other degeneration. The accumulation of calcium is apparently the primary result of the deficiency or toxicity.

There may be a correlation between the increase in water content of the liver and the hydropic degeneration observed microscopically by others,[9,12] and noted in a few of our sections. However, histological observations do not show the change as consistently as the dry weight determinations reported here. It is interesting to note that deficiencies of the known B vitamins do not alter the moisture content of the tissues;[13,14] see also Table II.

Histological studies have shown[15,16] that administration of sulfadiazine results in crystallization of the drug or its acetyl derivative in the kidney with consequent irritation and calcification. The increase in calcium in the kidneys may be related to this phenomenon. There is no sound basis for postulating any other mechanism for this effect of sulfadiazine.

The slight decrease in muscle calcium (Table III) of rats fed sulfasuxidine or sulfathiazole may also be a result of the disturbed calcium metabolism since the ration can readily supply the relatively small amount of calcium (in terms of total utilization) accumulated in the liver.

Summary. Rats receiving sulfasuxidine in a purified ration exhibit a marked increase in the calcium content of the liver within 4 to 8 weeks. The increased calcium content is apparently a primary metabolic defect and not a secondary calcification following necro-

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[13] Haldi, J., Giddings, G., and Wynn, W., *Am. J. Physiol.*, 1944, **141**, 83.

[14] Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 297.

[15] Lehr, D., and Antopol, W., *Urol. and Cutaneous Rev.*, 1941, **45**, 545.

[16] Sobin, S. S., Aronberg, L. M., and Rolnick, H. C., *Am. J. Path.*, 1943, **19**, 211.

sis. Rats fed sulfathiazole show the same syndrome but not as consistently. The calcium of the kidney and muscle of rats fed sulfasuxidine or sulfathiazole is not increased. The altered calcium metabolism of the liver can be reversed or prevented by feeding solubilized liver for 10 to 40 days. Rats receiving sulfadiazine have an increased calcium content of the kidneys but not of liver or muscle.

14963 P

The Urinary Excretion of Sulfadiazine Determined with Thiobarbituric Acid, a New Specific Reagent.*

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By means of the thiobarbituric acid reagent described in another connection,[1] 2-aminopyrimidine and its derivatives sulfadiazine and acetylsulfadiazine can be determined quantitatively, the latter being recovered from urine to within 1%. The specificity is such that no reaction was obtained with 15 other pyrimidines, with sulfamerazine, or with sulfanilamide, sulfapyridine, sulfacetamide, sulfathiazole, sulfasuxidine, sulfapyrazine, and sulfaguanidine, with the common constituents of blood and urine, or with 50 control urines.

Procedure. Unknown and standard solutions are prepared to contain 0.5 to 2 mg% of sulfadiazine in 0.2 N HCl. 1 ml of unknown, of standard, and of 0.2 N HCl (the reagent blank) plus 4 ml of thiobarbituric acid reagent[1] are heated on the boiling water bath in chromic acid-cleaned tubes for 45 minutes, evaporation being minimized. The solutions are then made up to 10 ml with water, and within several hours the unknown and standard are read through filter 520 against the reagent blank in the Evelyn photoelectric colorimeter. The Beer-Lambert law is obeyed. The molar extinction coefficients of sulfadiazine and acetylsulfadiazine are equal. Preliminary hydrolysis of the urine (40 minutes, 0.2 N HCl) does not affect the analysis.

Urines from 30 subjects taking sulfadiazine were examined. In 25, the thiobarbituric acid analysis (TBA) of *fresh* urine agreed with the Bratton and Marshall[2] free sulfadiazine (BM-F). But after aging the urine at 5° or 20°C for one week, TBA agreed with the Bratton and Marshall free plus acetylated, or total (BM-T). The same change was obtained in 30 minutes by treating the fresh urine with ascorbic acid or sodium hydrosulfite (final concentration 0.6%). Example: analysis of fresh urine, TBA 61 mg%, BM-F 60.5 mg%, BM-T 91; after treatment with ascorbic acid, TBA 91.5 mg%.

In 5 subjects, however, aging had no effect, TBA agreeing with BM-T from the start. Example: analysis of fresh urine, TBA 113.5 mg%, BM-F 68 mg%, BM-T 115 mg%.

The analysis of a mixture of fresh urines from *both* types of subject equalled the sum of their individual analyses. This indicates that the difference between the types is not due to the presence of an interfering substance in one of them.

Following the ingestion of acetylsulfadiazine, both types of subject reacted in the same manner. De-acetylation was negligible, aging was without effect, and TBA equalled BM-T in fresh urine. Example: analysis of fresh

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* Thanks are due the Rockefeller Foundation for a grant in aid of this work, and the American Cyanamid Co. and Sharp and Dohme, Inc., for generous gifts of chemicals.

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urine, TBA 81.5 mg%, BM-F 4 mg%, BM-T 81.5 mg%.

That the sulfonamides experience various metabolic changes including oxidations is well known,[3,4,5,6] though the precise nature of these has not been determined in all cases. The present work indicates that the pyrimidine ring in sulfadiazine experiences some change which blocks the TBA test and which can be reversed by reduction. However, when acetylsulfadiazine passes through the body, this change does not occur.

Summary. Thiobarbituric acid is a specific reagent for the determination of sulfadiazine (free or acetylated) among the sulfonamides because it reacts with the aminopyrimidine moiety. In most urines, however, the acetylated fraction which appears following the ingestion of sulfadiazine does not react unless the urine first be aged or be treated with a reducing agent. This suggests that the pyrimidine ring has been oxidized. Acetylsulfadiazine taken by mouth, when subsequently excreted in the urine, does not experience this change.

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14964

A Modification of the Technic for Determination of the Antistreptolysin Titer.*

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The determination of the antistreptolysin titer of human serum has proved to be a valuable tool for use in the study of hemolytic streptococcal infection and related disorders such as rheumatic fever. Its usefulness has been limited by certain technical difficulties inherent in the method most widely used, which was described by Coburn and Pauli,[1] as modified from Todd[2] and Hodge and Swift.[3]

Streptolysin or streptococcus hemolysin is unstable and is readily oxidized to an antigenically potent substance which is unable to hemolyze red blood cells. Previous technics have maintained a lysin for testing purposes in the active state by reduction with sodium hydrosulphite, followed by removal of oxygen in a vacuum, and storage under an oil seal in the cold room. This procedure is cumbersome and deterioration of the lysin occurs rapidly under any but the most favorable circumstances. Furthermore, it has been difficult to regularly produce a lysin of adequate potency because of variations in available culture medias and other factors.

Streptolysin, in the oxidized state, is stable and its hemolytic potency remains unchanged, even though it has been stored for many months in the refrigerator or for relatively long periods at room temperature before reduction. It may also be readily concentrated and partially purified.[4,5,6].

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* This investigation was aided through the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases, Preventive Medicine Service, Office of the Surgeon General, United States Army.

The work described in this paper was done under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development, and Stanford University.

[1] Coburn, A. F., and Pauli, R. H., *J. Exp. Med.*, 1935, **62**, 129.

[2] Todd, E. W., *J. Exp. Med.*, 1932, **55**, 267.

[3] Hodge, B. E., and Swift, H. F., *J. Exp. Med.*, 1933, **58**, 277.

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TABLE I.

Ml diluted lysin	0.35	0.30	0.25	0.20	0.15	0.10
Ml buffered cysteine	0.15	0.20	0.25	0.30	0.35	0.40

These facts suggested a modification of the method for the determination of the antistreptolysin titer of serum which has proved to be extremely satisfactory in routine use and to give consistently reproducible values. It is based upon the preparation of a concentrated streptolysin which is stored in the oxidized state. Reduction to the active form is carried out at the time that tests are to be done.

Method. Preparation of lysin. A strain[†] of group A hemolytic streptococcus known to produce large amounts of hemolysin is grown for 18 hours in a meat infusion broth containing .2% of dextrose, 2% of proteose peptone (Difco), and buffered by the addition, after autoclaving, of .2% of sodium bicarbonate as a sterile solution. The original inoculum should be a large volume of rapidly growing organisms in a liquid medium.

After incubation the streptococci are removed in a centrifuge, the Sharples high speed type being most satisfactory if large volumes are to be processed. The lysin may now be sterilized by filtration through the Seitz filter and used, but the volume is large and the hemolytic activity may be insufficient. It is, therefore, very convenient to concentrate and partially purify the material.

The clarified culture is brought to .57% saturation by the addition of 429 g per liter of ammonium sulphate at 20°C. The resulting precipitate is removed in the regular or Sharples centrifuge, and dissolved in a phosphate buffer at pH 7.0 ($Na_2HPO_4 \cdot 12H_2O$, 14.2 g; KH_2PO_4, 3.63 g per liter of distilled water). The final volume should be about 4% of that of the original culture. This viscous brown solution is transferred to cellophane sacs and dialyzed for 18 hours against running tap water. This procedure is associated with a considerable increase in volume.

Nine g of sodium chloride is added to each liter of the dialyzed material which is then sterilized by passage through the Seitz filter. After aseptic transfer to sterile tubes, it is ready for use.

Determination of combining unit. The determination of the antigenic and hemolytic properties of the concentrated lysin requires its reduction at the time of use. This is accomplished by mixing it with an active reducing agent prepared in the following manner: 1.6 g of sodium hydroxide is added to 1 liter of an isotonic buffer of a pH of 6.5 (NaCl, 4.2 g; KH_2PO_4, 3.17 g; $Na_2HPO_4 \cdot 12H_2O$, 3.58 g per liter of distilled water). At the time of testing .15 g of cysteine hydrochloride is dissolved in 25 ml of this alkaline buffer to make a M/25 solution of the active agent.

To determine the combining unit the concentrated lysin is diluted serially from 1:2 to 1:12. Samples of each dilution are then set up in small tubes according to the schedule in Table I.

Reduction is permitted to proceed for 10 minutes when 1 ml of a dilution of standard antistreptolysin, containing 1 unit per cc, is added to each tube, thoroughly mixed and incubated for 15 minutes in the water bath. At the end of this time, 0.5 ml of a 5% suspension of thrice-washed defibrinated rabbit red cells are added to each tube. It is desirable to wash and suspend the cells in the isotonic buffer of pH 6.5 without added sodium hydroxide. After further incubation for 45 minutes the tubes are removed from the water and centrifuged. That amount of lysin which has just failed to produce hemolysis is the combining unit. In this laboratory it is usually .15 ml of the 1.8 dilution of the concentrated material.

Determination of antistreptolysin titer. The antistreptolysin titer of sera may be determined by the method described by Coburn and Pauli,[2] using the freshly reduced lysin. If this is to be done, the concentrated lysin is

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[5] Herbert, D., and Todd, E. W., *Biochem. J.*, 1941, **352**, 1124.

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[†] The Richards strain has been used in this laboratory. It and a supply of serum of known antistreptolysin titer were supplied by Dr. Rebecca C. Lancefield.

TABLE II.

	Serum dilution											
	1:10		1:100					1:500				
Ml of diluted serum	0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2
Ml of isotonic buffer	0.2	0.8	0.0	0.2	0.4	0.6	0.7	0.0	0.2	0.4	0.6	0.8
Ml of reduced lysin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Unit value of each tube	12	50	100	125	166	250	333	500	625	833	1250	2500

first diluted to the degree determined to be suitable by the previous test with isotonic buffer. A further dilution in the buffered cysteine solution is then performed at the time of use, so that the combining unit will be contained in a final volume of 0.5 ml. Thus a concentrated lysin might be diluted 1:8, 15 ml of this material being then added to 35 ml of the reducing solution. The lysin is ready for use after 10 minutes of exposure to the reducing agent and does not deteriorate within 60 minutes.

Lysin prepared in this way may be used in the usual technics, but it has been most convenient to eliminate the two-stage procedure previously described,[1,3] and to determine the antistreptolysin titer of each serum in a single operation, by means of a simplification of a procedure originally suggested by Hodge and Swift,[3] which does not appear to have been widely applied. This may be accomplished by preparing initial serum dilutions in isotonic buffer of 1:10, 1:100, and 1:500, samples of each of which are studied according to the scheme in Table II.

After 15 minutes of incubation at 37°C in the water bath, .15 ml of the previously described suspension of rabbit red cells are added and the whole maintained at the same temperature for an additional 45 minutes. The last tube in which lysis has not occurred indicates the antistreptolysin titer of the serum. A control serum of known antibody titer should always be included as a check on the potency of the lysin.

Discussion. The modified technic for the determination of the antistreptolysin titer described above, based upon the reduction of a concentrated standard lysin at the time of use, has certain advantages over methods previously described.

The lysin stored in the refrigerator and the reducing agent in the dry form remain stable for very long periods of time and are immediately ready for use. The complicated technic involving removal of dissolved oxygen in a vacuum and storage under oil is eliminated, as is the hazard of premature oxidation of the lysin before use. In addition, the presence of the reducing agent in adequate amounts during the actual testing, guarantees that the lytic agent will remain active during the interval required for the completion of the procedure.

Concentration of the lysin, while not essential, provides a product of suitable potency, makes sterilization by filtration more simple and decreases the volume for storage and shipment.

Other investigators[2] have indicated that lysin, after reduction, must be "aged" in order to obtain a stable product of maximum potency. Careful study of the technic described here demonstrates that this is not the case, and that the material is ready for use after exposure to the reducing agent for 10 minutes.

The suggested modification in which the antistreptolysin titer is determined as a single-stage operation greatly reduces the time involved in the performance of the tests. Larger than usual decrements of diluted serum are used but the values in units obtained are suitable for any investigative purpose except under circumstances involving the study of sera of very high antibody titer, when further determinations with a different dilution system may occasionally be desirable.

Many human and animal sera have been studied by the technic described and the results have been most satisfactory. Certain sera used as controls have been repeatedly titrated by different technicians, using several lots of lysin, with errors consistently less than might have been expected as the result of the dilutions involved. It has also been possible to ship lysin to other laboratories and to establish the method there with comparable results.

Summary. 1. A modified technic for the determination of the antistreptolysin titer of serum has been described which involves the use of a concentrated lysin reduced to the active form at the time of use.

14965

Effect of Heparin and Dicoumarol on Thrombosis Induced in the Presence of Venous Stasis.*

Campbell Moses. (Introduced by C. C. Guthrie.)

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The effect of heparin[1,2,3] and more recently dicoumarol[4,5,6] on the course of experimentally induced venous thrombosis has been extensively studied. However, the methods of producing intravascular thrombi used have not always been uniformly successful. For the evaluation of the effect of anticoagulants upon thrombosis a method of consistently producing thrombi is essential. In a preliminary report[7] the author states that he found stasis a prerequisite for the consistent production of occluding thrombosis by the insertion of intravascular foreign bodies. Rabinovitch and Pines[3] also found it necessary to add stasis (by partial ligature of the vein) to the intimal damage produced by stretching the vein to insure thrombosis. In view of the effect of stasis on experimental intravascular thrombosis[7,8] and the generally held clinical opinion as to stasis in the pathogenesis of thrombosis,[9] the antithrombotic action of heparin and dicoumarol [3,3′-methylenebis (4-hydroxycoumarin)] was studied in the presence of venous stasis.

Methods. Rabbits were used as the experimental animal. Prothrombin concentrations were estimated by the method of Quick[11] using rabbit brain thromboplastin in both whole and 16% plasma.[10,12] Coagulation times were determined by the capillary tube method using free-flowing venous blood. In preliminary experiments[7] various sclerosing agents and the intravascular insertion of darning cotton and wool yarn failed to consistently induce thrombosis in the absence of stasis and congestion. Soaking the wool yarn in defibrinated blood was found to augment thrombus formation to some degree. In the method of producing thrombosis finally adopted white wool knitting yarn was soaked in defibrinated dog's blood, thoroughly dried, and all gross masses of dried blood removed by gently pulling the yarn between the fingers. It was then unraveled into single strands; 2 strands loosely wound together were used as an intravascular foreign body. The neck of rabbits anesthetized

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* The author is indebted to Dr. C. C. Guthrie and Dr. T. K. Kruse for much helpful criticism and to Mr. Boyd E. Kimberling for technical assistance.

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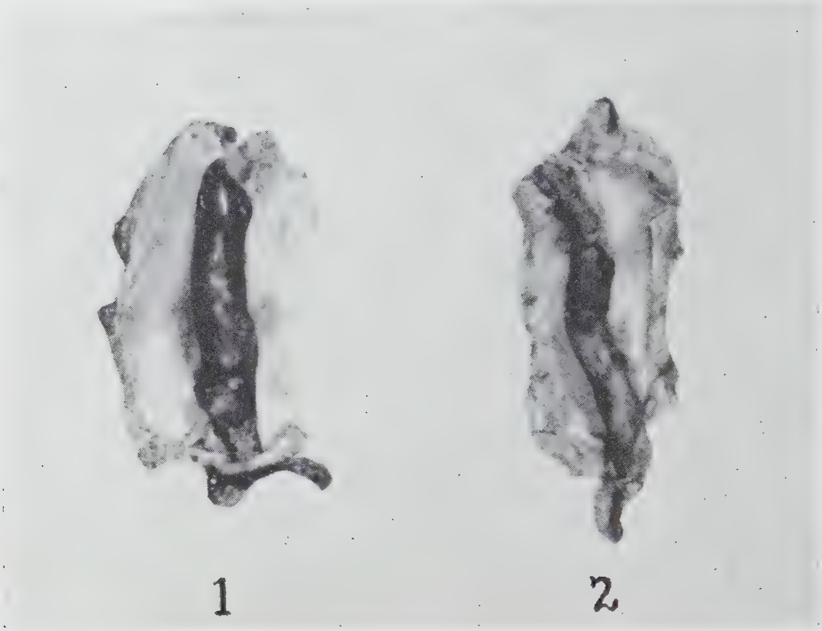

FIG. 1.
Thrombi produced in the jugular vein of rabbits by the insertion of blood-soaked wool yarn in the presence of stasis.

with nembutal intravenously, was shaved and a midline incision made to expose both jugular veins. The fascia was gently removed from these vessels from low in the neck to well above their bifurcation. A double strand of the blood-impregnated wool was threaded via a straight needle through 2 or 3 cm of the lumen. Temporary pressure with cotton sponges was used to control oozing following the venepuncture. A small (2 x 10 mm) metal clip was placed about the lower end of the exposed vein in such a manner that 3 or 4 respiratory cycles occurred before the vein emptied when it was digitally compressed above the foreign body. Great care was taken to insure the patency of the vein at this point. The wound was closed with a continuous cutaneous cotton suture. No dressing was applied, thus avoiding external compression of the vein.

Control Animals. In 6 animals 2 strands of blood-impregnated wool yarn were inserted on each side, and metal clips were placed about both jugular veins. In all of the 6 rabbits bilateral occluding red thrombi were found in 48 hours. (Fig. 1 and 2.) In 6 other rabbits the wool yarn was inserted bilaterally but the metal clips to slow the flow of blood were placed on only one side. Occluding red thrombi were present in 48 hours in all the veins constricted by a clip but in only 3 of the 6 veins to which no clip had been applied. The consistent occurrence of occluding thrombosis in veins treated in this manner in the presence of stasis and congestion and the failure of thrombosis to occur in one-half of the veins in which no stasis was induced emphasizes the importance of stasis in the production of experimental thrombosis by this method.

Heparinized Animals. Heparin was administered as the sodium salt intravenously in 10 mg doses twice daily to all animals in this series. Six rabbits received the first injection of heparin immediately before the bilateral jugular insertion of the wool yarn. Prior to heparin administration the coagulation time of all animals (from 3 to 5 minutes) was recorded twice daily for 2 days. Coagulation times obtained 6 hours after the last previous injection of heparin were consistently over 60 minutes. Of the 6 animals in this series one died of spontaneous hemorrhage 24 hours and one 36 hours after operation. At autopsy both animals showed gross gastrointestinal and intraperitoneal hemorrhage. The jugular veins in both animals contained firm red thrombi. The 4 remaining animals were autopsied 48 hours after operation; both jugular veins were found to be occluded by firm thrombi in every case. In 2 additional rabbits blood-impregnated wool yarn was inserted into both jugular veins, but partial constriction of the vein by a metal clip was omitted. Determination of coagulation times and the administration of 10 mg of heparin intravenously twice daily as noted were also carried out in these animals. After 48 hours the jugular veins of both animals contained small thrombi with the colorless characteristics of "white thrombi."[1]

Dicoumarolized Animals. Seven rabbits were used in this series of experiments. Control prothrombin and coagulation times were established in each animal on 2 successive days. Following this a single excessive dose of dicoumarol[†] (100 mg) was administered by mouth. Prothrombin times were determined daily until the animal died or was killed. When the prothrombin concentration reached 20% or less of the control value (usually within 48 hours) insertion of a double strand of wool yarn as described above was carried out. In 3 of this group of animals a metal clip was placed on only one jugular vein; in the other 4 animals metal clips were placed bilaterally using the precautions to insure incomplete obstruction outlined above. Three of the animals died within 36-48 hours after operation from generalized hemorrhage. All the remaining animals were examined 48 hours after operation. All the jugular veins that had been constricted with a metal clip contained red thrombi indistinguishable from those in the untreated control animals. Of the 3 veins which had not been constricted by a clip, 2 contained thrombi distinctly smaller than those of the untreated controls; the third vein contained a thrombus the same size as in the controls.

Summary and Conclusions. 1. A method for the consistent production of experimental intravascular thrombosis is described. 2. The important role of stasis in the pathogenesis of thrombosis is emphasized. 3. The results show that the administration of heparin and dicoumarol in adequate doses to delay coagulation was not sufficient to prevent the development of experimental intravascular thrombosis in the presence of stasis of the venous circulation as induced in these experiments.

[†] Supplied by Abbott Laboratories through the courtesy of Dr. J. F. Biehn.

14966

Increased Resistance of Carrot-Fed Rats to Carbon Tetrachloride.

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The increased resistance of carrot-fed rats to acute anoxia has been reported by Campbell[1,2,3] and later confirmed by Nelson *et al.*[4] Since there is considerable evidence suggesting that the necrosis of the liver which follows the administration of carbon tetrachloride may be due to tissue anoxia, rather than to a direct toxic effect of the halogen hydrocarbon on the liver cells, it was decided to determine if a carrot diet would increase the resistance of rats to carbon tetrachloride. The possible protective action of charcoal was also studied as Campbell had shown that a high charcoal diet also increased the resistance of rats to acute anoxia.

Rats weighing from 150 to 250 g were used for all of the experimental work. In each individual experiment animals of the same sex and of approximately the same size were used for both controls and experimental animals. The experimental animals were fed a diet of scraped raw carrots for a period of 7 to 10 days after which they and control animals on our stock diet were anesthetized with carbon tetrachloride in a previously described chamber.[5]

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[3] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1939, **29**, 259.

[4] Nelson, D., Goetzl, S., Robins, S., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 1.

The animals were kept in the chamber about an hour and three-quarters when mortality studies were being done and about an hour and a half when the degree of protection was to be based upon the histological changes in the liver. In the latter cases, the animals were killed approximately 24 hours after anesthesia, the livers were removed and a portion of the large left lobe taken for histological examination after staining with eosin and hematoxylin. As a general rule in the experiments designed to study survival, no food was given for the first 24 hours following anesthesia. After this time Rockland rat diet was fed to both groups. The difference in the animal's desire to eat was always very marked. Most of the carrot-fed animals began to eat immediately after the food was put in their cage, while few control animals ever showed any desire to eat at that time.

The control animals in all experiments unless otherwise specified were white rats from our stock colony and were fed on Rockland rat diet. This diet has been used in our laboratory for a number of years and it has been our experience that an hour or so of anesthesia, or a maximum of an hour and three-quarters in the gassing chamber, would usually produce sufficient liver damage to cause the death of about 50% of the untreated animals. Early in the fall of 1944 a definite increase in the resistance of our stock animals was noted. This increased resistance was so great that only a slight difference could be noted histologically between the livers of the control and carrot-fed animals. The reason for this increased resistance is unknown but it is probably the same factor or factors present in carrots which increases the animal's resistance to carbon tetrachloride. A personal communication from the manufacturer states that the concentration of vitamins A and E in the diet was increased in the spring of 1944 but gives no other change. It is very doubtful if the increased content of vitamins A and E can explain the results which we have obtained. However, since we purchase our food every 2 or 3 months, it is probable that the increased resistance noted coincided with our purchase of the improved diet.

On account of this disconcerting situation it was decided to work with rats raised on a diet of known composition.* This diet has been used successfully for years by Dr. R. J. Main of the Department of Physiology in raising rats for vitamin D assay. It is low in vitamin D. The animals received about 10 g of greens per rat per week, in addition to the above diet up to the beginning of the experimental period. The results obtained with the animals raised on this diet were in complete agreement with those obtained previously using the unimproved Rockland rat diet. Survival data using the different diets are shown in Table I. No comparative survival experiments were carried out using the improved Rockland diet on account of the slight difference noted histologically.

In addition to these mortality studies over 50 rats raised on unimproved Rockland diets were used for comparative histological examination of their livers following exposure to carbon tetrachloride. The degree of liver damage was always less in the carrot-fed animals than in the corresponding controls, the results being in complete agreement with what one would expect from the mortality studies.

Attempts at fractionation of the carrots have led to inconclusive results. A carrot extract made with 95% alcohol when evaporated to a thick syrup at room temperature with the aid of a current of air and mixed with the Rockland diet usually imparted definite protective action to the diet. This protective action was lost if the extract was allowed to stand for several weeks at a tem-

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[5] Forbes, J. C., and Neale, R. C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 319.

* Yellow corn meal 22%, wheat 19, wheat germ 4, soybean meal 19, dried skim milk 15, alfalfa 5, cotton seed oil 4, meat and bone meal 10, sodium chloride 1, calcium carbonate 0.5, and dried brewers yeast 0.5%.

TABLE I.
Effect of Carrot Feeding on the Resistance of Rats to Carbon Tetrachloride.

	No. of rats				
	Carrot fed		Controls		
Exp. No.	Lived	Died	Lived	Died	Control diet fed
1	4	0	0	4	Unimproved Rockland diet
2	3	0	2	1	,, ,, ,,
3	4	0	2	2	,, ,, ,,
4	6	0	1	5	,, ,, ,,
5	6	0	3	3	,, ,, ,,
6	4	0	2	2	,, ,, ,,
7	4	0	1	3	,, ,, ,,
8	8	1	2	2	,, ,, ,,
9	5	0	0	5	Low vitamin D diet
10	6	0	0	6	,, ,, ,, ,,
11	5	0	2	3	,, ,, ,, ,,
	55	1	15	36	

perature not exceeding 10°C. Ground carrots dried on a steam bath also were inactive. The addition of either carotene, alpha tocopherol, thiamine, niacin, riboflavin, calcium pantothenate, a folic acid concentrate plus biotin, vitamin A and D as in Haliver oil (Abbott) or a combination of vitamins A, D, and E as in a 1 in 3 mixture of Haliver oil and wheat germ oil to the unimproved Rockland diet or to the low vitamin D diet did not demonstrably affect the protective quality of either diet.

In all the experiments where the possible effect of charcoal was being studied, the animals were given a diet consisting of 25% Norite-A and 75% unimproved Rockland diet. After the rats were 8-10 days on this diet, they, along with the controls, were anesthetized with carbon tetrachloride as previously described. The animals were killed approximately 24 hours after the time of anesthesia, their livers removed, sectioned and histologically examined after suitable staining. Although the charcoal-fed animals, on the whole, showed less liver damage than the corresponding controls the degree of protection was definitely less than that obtained with the carrot diet.

Summary. Rats fed a carrot diet for 7 to 10 days are definitely more resistant to the hepatotoxic effects of carbon tetrachloride than corresponding animals fed either a well-balanced stock diet, or a low vitamin D but otherwise well-balanced diet.

Admixture of activated charcoal at a 25% level with the stock diet endowed it with some protective action. The effect, however, was much less than given by the carrot diet.

We are indebted to Charles C. Haskell & Co., Inc., Richmond, Virginia, for a grant in aid of this research.

14967 P

Homoplastic Ovarian Transplantability in the House Mouse.*

G. Gordon Robertson. (Introduced by A. D. Keller.)

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Offspring have been obtained from homoplastic ovarian transplants in guinea pigs by Castle and Phillips,[1,2,3] and in mice by this author.[4,5] The grafting technic consists of (1) exposure of the ovary of the host by a dorso-lateral incision in the abdominal body wall, (2) a small incision in the ovarian capsule as far distant from the Fallopian tube as possible, (3) complete removal of the ovary, (4) insertion of the donor ovary into the host ovarian capsule, and (5) suturing of the abdominal wall, but not the ovarian capsule.

Ovarian Transplantation Following Unilateral Ovariectomy in the Host. In 13 cases a single ovary was transplanted from a yellow mouse of the $101A^Y$ inbred line into an agouti litter mate, the host differing from the donor in but a single genetic character. The agouti hosts were later test mated with agouti males. Since the yellow factor is dominant to agouti any yellow offspring resulting from such a mating would indicate a successful graft, as follows:

$$\begin{array}{ccc} A^{Y}A & \times & A\ A \\ \text{agouti host with yellow ovary} & & \text{agouti test male} \\ & \downarrow & \\ 1\ A^{Y}A & : & 1\ A\ A \end{array}$$

In every instance the only offspring obtained from the test matings were agouti, thus indicating that the grafts were not successful.

Ovarian Transplantation Following Bilateral Ovariectomy in the Host. In 38 cases one ovary of an agouti mouse of the $101A^Y$ line was removed. Seven to 10 days later the remaining hypertrophied ovary was removed, and in its place an ovary from a yellow litter mate was inserted into the capsule. The agouti hosts were later test mated with agouti males. Eighteen of the 38 agouti hosts had yellow young in litters resulting from these matings, thus demonstrating functional grafts in 18 cases.

In an attempt to analyse the problem of homoplastic ovarian transplantability both ovarian sites of all 38 mice in the above group were examined histologically. This consisted of a study of serial sections of the region of the grafting operation as well as the site of the initial ovariectomy. Care was taken to remove for sectioning enough of the surrounding area to eliminate any possibility of doubt as to the presence or absence of ovarian tissue. The results, along with the results of transplantation after unilateral ovariectomy, are shown in Table I. In the 8 cases of incomplete bilateral ovariectomy previous test matings had indicated unsuccessful grafts. Of the remaining 30 females ovarian tissue was entirely absent in 12 cases. In each of the 18 successful cases the only ovarian tissue present was the hypertrophied donor ovary in the graft site.

Discussion. It is evident that in this work homoplastic ovarian grafts were successfully established and maintained only after complete bilateral ovariectomy in the host. Thus the results are entirely compatible with Halsted's "law of endocrine deficiency"[6] which states that there must be a deficiency of an endocrine principle before transplants supply-

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* This work was begun under the supervision of Profs. J. S. Nicholas and D. F. Poulson at Yale University.

[1] Castle, W. E., and Phillips, J. C., *Science,* N. S., 1909, **30**, 312.

[2] Castle, W. E., and Phillips, J. C., Carnegie Institution of Washington, 1911, Publ. No. 144.

[3] Castle, W. E., and Phillips, J. C , *Science,* N. S., 1913, **38**, 783.

[4] Robertson, G. G., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 302.

[5] Robertson, G. G., *J. Exp. Zool.,* 1942, **89**, 197.

[6] Halsted, W. S., *J. Exp. Med.,* 1909, **11**, 175.

TABLE I.
Relationship of Homoplastic Ovarian Transplantability to the Type of Ovariectomy in the Host.

Type of Ovariectomy	No. of attempted grafts	Unsuccessful grafts	Functional grafts
Unilateral	13	13	0
Incomplete bilateral	8	8	0
Complete bilateral	30	12	18

ing that principle can be successful. This has been confirmed by results of autoplastic transplantations of parathyroids (Halsted),[6] adrenal glands (Ingle and Higgins),[7] thyroid (Ingle and Cragg),[8] and ovaries (Lane and Markee),[9] and homoplastic transplantations of adrenal glands (Pomerat *et al.*).[10] In the present work homoplastic ovarian transplantations were successful only when there was a complete absence of host ovarian tissue. Therefore, the results are in accord with the concept that creation of a deficiency of the ovarian principle is necessary for the establishment and maintenance of homoplastic ovarian grafts.

Summary. Functional homoplastic ovarian grafts in mice became established only after complete bilateral ovariectomy in the host. This is in agreement with Halsted's "law of endocrine deficiency" since, in this series of experiments, the transplants were successful only in the absence of host ovarian tissue.

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[7] Ingle, D. J., and Higgins, G. M., *Endocrinology*, 1938, **22**, 458.

[8] Ingle, D. J., and Cragg, R. W., *Endocrinology*, 1939, **24**, 550.

[9] Lane, C. E., and Markee, J. E., *Growth*, 1941, **5**, 61.

[10] Pomerat, C. M., Breckenridge, C. G., and Gordon, L., *Endocrinology*, 1944, **34**, 60.

14968

Acetylcholine in the Light and Dark Adapted Frog Retina.

DEXTER M. EASTON. (Introduced by F. L. Hisaw.)

From the Biological Laboratories, Harvard University.

Like central nervous tissue of which it is a derivative, the vertebrate retina has been shown to contain acetylcholine (Ach)[1,2,3,4] and cholinesterase.[5] That Ach may affect the retina has also been demonstrated, von Studnitz and Kosarov[6] having observed a contraction of the cones and an expansion of the rods at concentrations of 10^{-4} and 10^{-8} Ach upon both light and dark exposure. In the excised eye, Therman[2] has found that the application of Ach causes an irreversible depression of excitation as measured by diminished retinal potentials (an effect enhanced by eserine).

Two attempts to demonstrate a possible functional significance of retinal Ach by comparing the amounts of Ach in frog retina under conditions of light and dark adaptation have resulted in completely contradictory results. Therman,[2] using the leech muscle and the frog heart as test objects, found under both conditions of adaptation 6 γ Ach/g of retina extracted with trichloracetic acid. Lange,[4] extracting with bicarbonate-free frog Ringer, and assaying with the leech muscle, found, after complete light adaptation of 10 frogs,

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[1] Loewi, O., and Hellauer, H., *Arch. f. d. ges. Physiol.*, 1938, **240**, 769.

[2] Therman, P. O., *Acta Soc. Sci. fenn.*, Ser. B, 1938, **2**, 5.

[3] Chang, H. C., Lee, L. Y., and Li, T. A., *Chinese J. Physiol.*, 1941, **16**, 373.

[4] Lange, V., *Ztschr. f. Physiol. Chem.*, 1943, **279**, 73.

[5] Anfinsen, C. B., *J. Biol. Chem.*, 1944, **152**, 267.

[6] Studnitz, G. v. and Kosarov, W. G., *Z. f. vergl. Physiol.*, 1942, **29**, 418.

TABLE I.

Exp.	Time adapted, hr	Frog	Wt 2 retinas, mg	Total Ach, γ/g
Acetylcholine Content of Dark-Adapted Retinas.				
10-12	48	A	29	5.0
		B	23	4.0
10-9	115	A	37	2.8
		B	31	3.2
		C	22	3.0
10-4	36	A	28	4.0
		B	31	2.8
		C	29	2.0
9-30	42	A	23	4.0
		B	23	4.8
				Avg 3.6
Acetylcholine Content of Light-Adapted Retinas.				
10-12	48	A	36	3.0
		B	28	3.0
10-11	20	A	35	(1*)
		B	31	5.0
10-4	2	A	36	3.0
		B	28	3.0
		C	28	3.0
10-3	2	A	33	2.8
		B	24	3.5
		C	24	2.5
9-30	1.5	A	29	4.0
		B	23	4.5
				Avg 3.4

* Not included in the average.

30 γ Ach/g of tissue in the 20 pooled retinas; after complete dark adaptation of an equal number, 300 γ Ach/g.

The following experiments were performed in an attempt to resolve these differences.

Methods. The eyes of dark-adapted frogs (*Rana pipiens*) were prepared throughout under ruby light, those light-adapted were prepared under bright light. The cornea with the lens was cut from the excised eye; the remaining portion was then immersed in eserinized (*ca.* 1:50,000 by weight) bicarbonate-free Ringer solution, the more easily to tease out the retina. Blotting the retinas with filter paper, before weighing, removed excess moisture. The retinas from each frog were ground in eserinized Ringer solution, and the volume of the suspension was increased to secure a final proportion of 10 mg of tissue per cubic centimeter of fluid. Total Ach was determined one hour or more after the addition of *ca.* 0.1 cc of normal HCl. The suspension was brought to pH 5-6 with normal NaOH before testing. Ach determinations were made with the heart of *Venus mercenaria,* according to the suggestion of Prosser.[7] Comparative experiments on other material reveal that the *Venus* heart is more sensitive than the leech muscle, and equally as accurate a test object for the quantitative determination of Ach.[8] The sensitivity of the heart enables one to make several determinations of Ach in such small amounts of tissue as 2 frog retinas. One cubic centimeter of the suspension was added to the heart bath of oxygenated sea water (total volume 10 cc), and the resultant depression matched, after a 15-minute recovery period, with that of a known concentration of Ach, or with another retinal extract.

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[7] Prosser, C. L., *Biol. Bull.*, 1940, **78**, 92.

[8] Welsh, J. H., unpublished observations.

Results. In one series of experiments (Table I) 2 or 3 frogs were dark-adapted for an approximately equal length of time in each experiment. Two or 3 were also light-adapted for the same or different lengths of time. In 3 of the experiments, determinations of Ach in both light and dark retinas were made on the same heart.

The values of total Ach given in Table I are of the same order of magnitude as those reported by other authors except Lange. Chang *et al.*[3] found about 2 γ Ach/g of tissue in the normal dog retina extracted with alcohol. After an eye had been blindfolded for 7 to 220 days they found an Ach content averaging 1.3 γ/g. This was interpreted as being a consequence of "functional disuse." During the first few days of blindfolding, when any effect would presumably be that of "dark adaptation," the values were not significantly different from "normal." Loewi and Hellauer[1] reported 5-6 γ of Ach/g of cattle retina.

The data in Table I indicate: (1) fairly good correspondence in any one experiment between individuals similarly adapted: (2) length of period of adaptation does not consistently alter the Ach content; (3) the difference in Ach content of light- and dark-adapted retinas is not significant.

During the course of a given assay, the sensitivity of the heart may decline. The last of eight or ten records is not, therefore, directly comparable with the first, although absolute values, as determined by direct matching with known Ach concentrations, will generally agree fairly closely. In order to bring out more clearly any light-dark differences, another series of experiments was conducted, in each of which 2 pooled dark-adapted retinas were matched several times, at different dilutions, against 2 pooled light-adapted retinas. Several direct matchings brought out no more variation than was found between individuals exposed to the same conditions of adaptation.

These experiments, though constituting an affirmation of Therman's failure to find any differences in Ach content of light and dark retinæ, do not necessarily prove that changes in retinal sensitivity may not be associated with changes in Ach content. The range of experimental error of the assay method is no less than 10%, even under the best conditions, and a difference of such magnitude might very well be of physiological significance.

Summary. Extraction of frog retinas in acidified Ringer solution reveals an average Ach content of 3.4 γ/g in light-adapted and 3.6 γ/g in dark-adapted frogs. Direct comparison of Ach activity in extracts of light- and dark-adapted retinas, without reference to absolute magnitudes, also reveals no difference greater than the error of the method. The lack of significant difference is in accord with the work of Therman, but not with that of Lange who finds a tenfold increase upon dark adaptation.

I wish to express my thanks to Dr. John H. Welsh for his assistance and encouragement in this work.

14969 P

Antibiotic Action of Trypsin Inhibitors.

I. Arthur Mirsky and George Foley

From the AAF Rheumatic Fever Control Program, the House of the Good Samaritan, and Department of Preventive Medicine and Epidemiology, Harvard Medical School, Boston.

In a previous communication[1] evidence was presented which demonstrated that the *in vitro* action of hemolytic streptococcal fibrinolysins was inhibited in the presence of protease inhibitors ("Trypsin-inhibitor" of Northrop and Kunitz[2] and soybean "antitrypsin"[3]). Irrespective of whether streptococcal fibrinolysin is an enzyme or, as has been suggested recently, an "activator" of human serum protease (tryptase), it is a definite metabolic product of streptococci which is believed to play some role in virulence of infection. Consequently, it became of interest to investigate *in vitro* the action of protease inhibitors on hemolytic streptococci and other bacteria.

0.1 ml of suitable dilutions of an 18-hour culture of the microörgnisms was transferred to 0.9 ml saline in which was dissolved from 10 to 50 mg of crystalline "trypsin-inhibitor" compound, making a total concentration of from 1 to 5% of the inhibitor in the solution. Dr. M. Kunitz provided some of the crystalline compound used in this study. Since the "trypsin-inhibitor" compound contains at least 50% $MgSO_4$, controls were set up with a similar concentration of MgSO. The tubes were incubated at 37°C and at various intervals 0.1 ml was transferred to 1 ml broth and to 10 ml blood agar, a pour plate being made from the latter. Twenty-four hours later, the broth and plates were examined. Various dilutions of the broth culture were then transferred to a constant amount of blood agar and a pour plate prepared, a colony count being made 24 hours later.

It was observed that in concentrations of from 1 to 5% of crystalline trypsin inhibitor $MgSO_4$ compound, growth of hemolytic streptococci did not occur or was markedly depressed after incubation of from 4 to 8 hours. Fig. 1 depicts a growth curve of such an experiment with streptococci, the curve being based on colony counts made on pour plates of suitable dilutions prepared at stated intervals and incubated 18-24 hours at 37°C. Table I depicts another typical experiment.

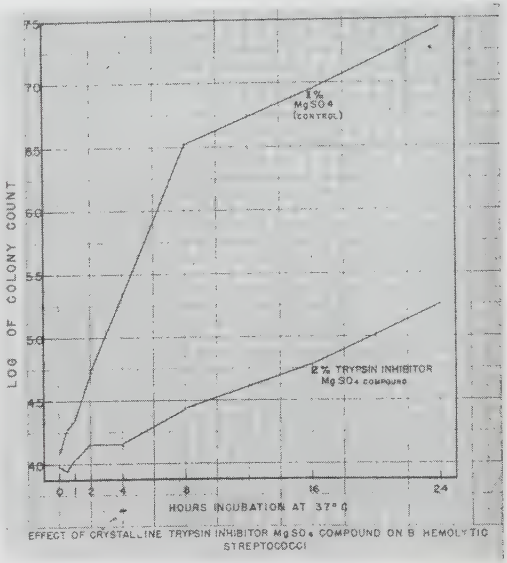

Fig. 1.

Studies with soybean "antitrypsin" preparations were uniformly negative, presumably due to the lack of purity for in most instances an actual stimulation of growth could be demonstrated. Relatively crude preparations of pancreatic trypsin-inhibitor gave results similar to those of the crystalline inhibitor.

In view of the observations with streptococci other microörganisms were studied. *Staphylococcus aureus* and *Escherichia coli* were utilized for these further studies. The results were identical with those obtained with streptococci

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[1] Mirsky, I. A., *Science*, 1944, **100**, 198.

[2] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932-33, **6**, 267.

[3] Ham, W. D., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

TABLE I.
Effect of Trypsin-Inhibitor on B Hemolytic Streptococci.

Incubation period before plating, hr	Colony count*	
	Trypsin-inhibitor $MgSO_4$ compound 5%	$MgSO_4$ 5%
2	†	†
4	656	†
6	23	†
8	0	†

* 0.1 cc of 1:1000 dilution of 18-hour culture removed at stated intervals after incubation with test substance and transferred to blood agar pour plates and incubated at 37°C for 24 hours.

† Too many to count.

in that inhibition of growth ensued on incubation with pancreatic trypsin-inhibitor.

In an excellent study of the effect of trypsin inhibitor on bacterial growth, Grob[4] demonstrated that growth was "somewhat retarded" in the presence of the inhibitor. He concluded that the trypsin-inhibitor had no bacteriostatic action but affected growth through the prevention of proteolysis in the medium. However, he could not demonstrate any action on *Staphylococcus aureus*. Our studies suggest that the inhibitor can be bacteriostatic when employed in adequate concentration (Table I).

It is obvious that the trypsin-inhibitor compound is not nearly as potent an antibiotic agent as is penicillin and other antibiotics. However, the relative ineffectiveness of penicillin on *E. coli* and the fact that trypsin-inhibitor affects *E. coli* to the same degree as hemolytic streptococci suggests a greater non-specificity of the antibiotic action of the protease inhibitor.

Unpublished data reveal that penicillin has a weak but definite antiproteolytic action as do Actinomycin A and Tyrothricin.[5] It is probable that the efficacy of penicillin as an antibiotic agent lies in its ability to inhibit enzymes other than the proteases. On the other hand, the effect of trypsin-inhibitor can be attributed solely to its inhibition of the bacterial proteases since it has been observed that this protease inhibitor is not specific but can inhibit a number of proteolytic enzymes. Further, the fact that synthetic detergents which are antiproteolytic are also germicidal lends support to this hypothesis.

The significance of the above observations and those of Grob lies not so much in the demonstration that the protease inhibitor has an antibiotic action as in the suggestion that the rise of the antiprotease titer of blood which occurs in a variety of pathologic states in man may play an important role in "resistance" to infection. In this respect the role of numerous other anti-enzymes known to be present *in vivo* merits intensive investigation.

Summary. Trypsin-inhibitor has antibiotic properties.

We wish to express our appreciation of the courtesies and facilities extended by Dr. T. Duckett Jones and the staff of the House of the Good Samaritan, and of our indebtedness to Col. W. Paul Holbrook for his interest and cooperation.

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[4] Grob, D., *J. Gen. Physiol.*, 1943, **26**, 431.

[5] Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 163.

14970

Alloxan Administration to the Duck.

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One of the perplexing problems in carbohydrate metabolism is the fact that pancreatectomy does not result in the development of diabetes mellitus in the duck or chicken. Further, the administration of crude saline extracts of the anterior pituitary gland to such

TABLE I.
Alloxan Administration to the Duck.

Alloxan, mg/kg intravenously	Blood sugar, mg %				Survival, hr
	Avg initial	Max. first 24 hr	Max. second 24 hr	Max. after 48 hr	
715	104	263	—	—	20
575	97	—	—	—	2
360	115	252	—	—	20
300	118	127	—	—	24 to 36
250	130	168	107	—	36 to 48
200	133	125	134	112	
150	108	126	123	118	
100	105	116	102	122	
50	114	100	107	110	

depancreatized birds does not result in the hyperglycemia observed in other species.[1] Estrogens, which cause a marked lipemia in fowl, are likewise without effect in producing hyperglycemia.[1]

It has been demonstrated that islet tissue is present in the fowl's pancreas and that appreciable amounts of insulin can be extracted from that organ.[2] Nevertheless, it is possible that aberrant islet tissue may be present in other parts of the body, and for that reason removal of the pancreas might not produce diabetes. Consequently, we deemed it of interest to examine the effect of alloxan in this species, since the administration of this compound produces necrosis of the β cells of the Islets of Langerhans and diabetes in all species that have been studied hitherto, including the pigeon.[3]

A variety of ducks (white, mallard, canvasback) were employed in this study. The dosage of alloxan administered as a 5% solution in a single intravenous injection varied from 50 mg to 750 mg per kilogram. Blood samples were drawn at frequent intervals from the wing veins and the sugar concentration determined by Nelson's modification of the Somogyi-Shaffer-Hartman method.[4]

The intravenous administration of more than 200 mg alloxan per kilogram resulted in death of the birds in from 3 to 48 hours. No constant blood sugar response was observed when such toxic doses were employed. Thus, some animals developed an immediate hyperglycemia which culminated with the death of the fowl in about 12 hours while others developed no hyperglycemia and died at variable periods up to 48 hours. In no instance was hypoglycemia noted.

The administration of smaller doses of alloxan did not produce any significant change in the blood sugar concentration (Table I).

Sections of the pancreas taken from animals sacrificed at various intervals after the injection of alloxan revealed that those animals which lived more than 12 hours developed islet lesions irrespective of the dose employed. Thus, the pancreas from one duck which was sacrificed 9 days after receiving 50 mg alloxan per kilogram, and which at no time developed hyperglycemia showed extensive necrosis of the islets. On the other hand, the pancreas of another duck which died within 2 hours after the injection of 750 mg alloxan per kilogram showed no significant deviation from the normal.

From the preceding it may be concluded that the duck, like other species is susceptible to the action of alloxan on the Islets of Langerhans. The fact that a permanent hyperglycemia did not result suggests that the duck has no extrapancreatic Islets of Langerhans. This is supported by the fact that the injection of 200 mg alloxan per kilogram to a depancreatized duck did not produce any change in the blood sugar level. Consequently, it can now be reiterated more definitely that the duck does not need insulin to maintain its carbohydrate metabolism, implying thereby the existence of regulatory mechanisms which differ markedly from those of other species. That a similar situation may exist in the chicken is indicated by our experience with two chickens which, after alloxan administration, showed no abnormality of the blood sugar level.

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[1] Mirsky, I. A., Nelson, N., Grayman, I., and Korenberg, M., *Am. J. Physiol.*, 1941, **135**, 223.

[2] Redenbaugh, H. E., Ivy, A. C., and Koppanyi, T., PROC. SOC. EXP. BIOL. AND MED., 1926, **23**, 756.

[3] Goldner, M. G., and Gomori, G., *Proc. Am. Diab. Assn.*, 1944, 4.

[4] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

Summary. Alloxan does not produce diabetes mellitus in the duck in spite of the fact that the drug does produce necrosis of the Islets of Langerhans.

We wish to acknowledge our appreciation of the cooperation and facilities extended by Dr. Kenneth G. Kohlstaedt, Director, and the staff of the Lilly Laboratory for Clinical Research, and our indebtedness to Dr. A. Nettleship for the histologic examination of the pancreases.

14971

Influence of Pantothenic Acid Deficiency on Resistance of Mice and Rats to Experimental Pneumococcal Infection.*

HARRY G. DAY AND L. S. MCCLUNG.

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Several recent reports are concerned with the effects of pantothenic acid deficiency in animals on their resistance to various infectious agents. Becker, Manresa, and Johnson found that pantothenic acid increases the production of oöcysts by rats infected by *Eimeria nieschulzi*[1] and reduces the efficiency of ablastic action of pantothenic acid-deficient rats infected with *Trypanosoma lewisii*.[2] According to Trager[3] the survival of *Plasmodium lophuræ in vitro* is favored by the presence of pantothenic acid. Lichstein, Waisman, Elvehjem, and Clark[4] reported that young, pantothenic acid-deficient, Swiss mice show increased resistance to Theiler's encephalomyelitis virus, but little or no change in resistance to the Lansing strain of poliomyelitis. West, Bent, Rivera, and Tisdale,[5] using 15 animals, claimed that pantothenic acid deficiency greatly increased the resistance of the rat, infected by nasal insufflation, to a strain of Type I pneumococcus. Since these organisms require pantothenic acid for growth, West *et al.* postulated that the apparent increase in resistance was due to inability of the pathogenic organisms to secure sufficient pantothenic acid from the host rats to permit maximal growth. Robinson and Siegel[6] did not find a change in resistance to experimental pneumococcal pneumonia in 20 young adult rats restricted to a pantothenic acid-deficient diet as compared to the resistance of control animals which were fed the vitamin.

Wooley and Sebrell[7] reported that young mice deficient in riboflavin or thiamine are more susceptible to Type I pneumococcus by nasal insufflation than controls which received these vitamins. Robinson and Siegel,[6] using 20 young adult rats, appeared to confirm these findings for thiamine.

An important problem in investigations of

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* Supported by a grant from the Graduate Research Fund of Indiana University. The authors are indebted to Miss Ruth Toabe and Mr. David K. Barnes for technical assistance.

[1] Becker, E. R., Manresa, M., Jr., and Smith, L., *Iowa State Coll. J. Sci.*, 1943, **17**, 257; *Chem. Abstr.*, 1943, **37**, 1478.

[2] Becker, E. R., Manresa, M., Jr., and Johnson, E., *Iowa State Coll. J. Sci.*, 1943, **17**, 431; *Chem. Abstr.*, 1943, **37**, 3801.

[3] Trager, Wm., *J. Exp. Med.*, 1943, **77**, 411.

[4] Lichstein, H. C., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 3.

[5] West, H. D., Bent, M. J., Rivera, R. E., and Tisdale, R. E., *Arch. Biochem.*, 1944, **3**, 321.

[6] Robinson, H. J., and Siegel, H., *J. Infect. Dis.*, 1944, **75**, 127.

[7] Wooley, J. G., and Sebrell, W. H., *U. S. Pub. Health Rep.*, 1942, **57**, 149.

TABLE I.
Effect of Varying Degrees of Pantothenic Acid Deficiency on the Resistance of Young Mice to Type I Pneumococcus.

Diet No.	No. of mice	Days on diet before inoculation	Wt increase per mouse, g	% dying during first 96 hr after inoculation	Avg survival time, hr
1[*]	10	7	2	70	38
2[†]	10	7	2	100	36
1	25	14	2	88	39
2	26	14	3	73	45
1	32	19	2	60	39
2	19	19	4	53	38
1	16	21	1	100	37
2	18	21	5	100	35
1	31	26	3	52	37
2	40	26	9	60	40
1	26	27	2	54	52
2	30	27	7	80	51
1	35	28	2	88	40
2	39	28	7	93	38
Summary.					
1	175	22	2	71	41
2	182	22	6	77	41

[*] Diet 1: Pantothenic acid deficient.
[*] Diet 2: Control diet (calcium pantothenate added).

the effect of diet on resistance to pneumococcal infection is the mode of infecting the animals. Nasal insufflation is extremely tedious and secondary injuries to the animals may result. An additional disadvantage is the fact that all animals may not take into the lungs comparable numbers of organisms even though caution is observed in the administration of the infecting dose. Direct introduction of the organisms into the bronchial tree by injection into the exposed tracheæ, as practiced by Robinson and Siegel, is less likely to produce secondary injuries but it is obviously troublesome. In the investigation to be reported, all animals were inoculated by intraperitoneal injections. Although this may not be the ideal route for production of experimental pneumonia it can give rise to a lethal infection and has been used extensively with mice. For example, Buck and Schnitzer[8] inoculated their animals by intraperitoneal injection to study the synergistic effect of penicillin and anti-pneumococcus serum.

Experimental. Weanling piebald rats from our own colony and young white mice purchased from the Harlan Small Animals Industry, Cumberland, Indiana, were used.

The pantothenic acid-deficient diet (Diet 1) contained: casein (vitamin-free), 18; glucose (Cerelose), 68; hydrogenated vegetable oil (Crisco), 7; solvent-extracted wheat germ oil,[†] 2; percomorph liver oil, 2 drops; salts No. 12,[9] 4; and vitamin mixture, 1. The vitamin mixture[‡] was composed of thiamine hydrochloride, 1 mg; pyridoxine hydrochloride, 1 mg; riboflavin, 2 mg; nicotinic acid, 10 mg; *p*-aminobenzoic acid, 20 mg; inositol, 20 mg; choline chloride, 120 mg; and glucose, 826 mg. The pantothenic acid-added diet (Diet 2) was identical to Diet 1 except that the vitamin mixture contained, in addition to the vitamins listed, 50 mg of calcium pantothenate, and a corresponding reduction in the content of glucose.

Sex and initial weight were considered in distributing the animals between Diets 1 and 2. Litter membership was also considered in the experiments with rats. This factor was not controlled with the mice since the various litters could not be distinguished in the shipments received from the breeder. The mice were kept in groups of 5 in roomy screen-bottomed cages; the majority of the rats were kept in individual cages. Tap water and food were permitted *ad libitum*. By means of air conditioning equipment, the temperature of the animal room was not allowed to exceed 82°F.

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[8] Buck, M., and Schnitzer, R. J., *Arch. Biochem.*, 1944, **5**, 153.

[†] The wheat germ oil was kindly furnished by Mr. Ezra Levin, VioBin Corporation, Monticello, Illinois.

[9] Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245.

[‡] Most of the synthetic vitamins were furnished through the courtesy of Dr. D. F. Robertson, Merck and Co., Rahway, New Jersey.

Both rats and mice restricted to Diet 1 showed the typical effects of pantothenic acid deficiency. Animals fed Diet 2 grew well and appeared normal. The effects on growth are indicated in Table I.

At intervals ranging from 19 to 38 days after restriction to the experimental diets, the rats were injected intraperitoneally with 0.2 ml of a 10^{-2} dilution of Type I pneumococcus.§ The dilutions, prepared in physiological saline solution, were made from a 6-8 hour culture grown in heart infusion broth enriched with tryptone (1%), glucose (0.2%), and fresh rabbit blood (0.2 ml per 8 ml of medium). At intervals the culture was passed through mice to maintain the virulence. The mice were injected intraperitoneally at intervals of 7 to 28 days after restriction to the experimental diets. The inoculum was prepared as described above and the dosage was 0.5 ml of a 10^{-6} or 10^{-7} dilution. All animals were observed frequently during a 4-day period following inoculation. Heart blood cultures on blood agar were made from a large number of the animals which died within this period to confirm the fact that death was due to the inoculated organism. Pneumococci were present in all samples which were plated.

The results of the experiments on rats may be summarized as follows: A total of 29 (74.3%) of the 39 rats on Diet 1 (pantothenic acid-free) died whereas 25 (69.3%) of the 36 animals on Diet 2 (control) succumbed. Although the percentage figure of the animals dying which were on the experimental diet is slightly higher than the value for those on the control diet, it is doubtful that the difference is statistically significant. Certainly, in so far as these data may be interpreted, there is no marked change in the susceptibility to the pneumococcus in rats showing pantothenic acid deficiency.

With respect to the mice, 125 (71%) of 175 on the pantothenic acid-deficient diet succumbed in contrast to 141 (77%) of the 182 on the control diet (Table I). This does not appear to be a significant difference. However, as shown in Table I, the animals were inoculated at intervals ranging from 7 to 28 days after being started on the test diets. Although there is no survival difference between the experimental and control mice inoculated between the 7th to 21st days (77% fatalities in the pantothenic acid-deficient group as compared with 78% in the controls), it may be significant that in the groups of mice restricted to the diets 26 to 28 days before inoculation 66% of the pantothenic acid-deficient animals died whereas 77% of the controls failed to survive.

Summary. A study of the resistance to the intraperitoneal injection of Type I pneumococci of 39 rats showing pantothenic acid deficiency in contrast to 36 controls fed pantothenic acid failed to reveal a significant change in the susceptibility of the experimental group. Similarly, no marked change in susceptibility was observed in 175 mice showing pantothenic acid deficiency in contrast to the survival percentage of 141 controls receiving the vitamin (normal animals). However, the difference is great enough to warrant the conclusion that acute pantothenic acid deficiency in mice may slightly increase the resistance to Type I pneumococcus. Certainly the results give no indication of decreased resistance to this organism in pantothenic acid deficiency.

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§ This culture was received through the courtesy of Dr. H. M. Powell of the Eli Lilly Research Laboratories, Indianapolis, Indiana.

14972 P

The Antibiotic Action of *Tillandsia usneoides* (Spanish Moss).

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It is now well established that certain bacterial organisms, fungi and algæ[1] produce antibiotic substances for various bacteria and fungi. Extracts of certain plants,[2,3] including lichens, and the heartwood of the Western Red Cedar[4] also have recently been shown to possess inhibitory properties. So far as we know, however, no epiphyte has previously been tested for inhibitory activity. The following is a report of tests that demonstrate that extracts of *Tillandsia usneoides* (Spanish Moss), an epiphyte, are also inhibitory to certain organisms.

The *Tillandsia* used in this work was taken from trees in the neighborhood of Charleston, S.C. We found that alcoholic, acetone, or chloroform extracts of this plant were frequently inhibitory to *Staphylococcus aureus.* On further study, we discovered that most of the antibiotic substance was present in certain brownish parts of the plant, and that preparations of relatively high potency could be obtained by subjecting these parts to extraction with acetone or chloroform.

Methods. The method which consistently gives the most potent extracts is as follows: The plant is cut up into fragments of 0.5 to 1.5 cm in length. In order to separate the brown from the green inactive parts, the fragments are stirred thoroughly in 95% alcohol. The heavier green fragments sink to the bottom of the container, while the brown lighter ones rise to the surface of the liquid and may be skimmed off and dried at 37°C. The weight of this dried material is approximately one-tenth of that of the original plant. The material is then shaken with acetone or chloroform for several hours, and filtered through one layer of gauze. The acetone or chloroform extract thus obtained is evaporated off at room temperature. The slight amount of brownish or greenish yellow residue on the evaporating dish is taken up in 2 cc of alcohol for every gram of brown material originally extracted. The cloudy fluid obtained is centrifuged, and the clear olive green or yellow supernatant thus obtained is tested for antibiotic activity. The brown Tillandsia fragments can be re-extracted several times with decreasing yields of inhibitory substances.

Most of the tests were carried out against *Staphylococcus aureus* H. (Oxford strain). A serial dilution method was employed similar to that used by McKee, Rake, and Menzel.[5] Bacto-nutrient broth was used throughout these experiments. The highest dilution that completely inhibited growth in two days at 37°C was taken as the endpoint. One inhibiting unit of the Tillandsia extract was arbitrarily taken as the minimum quantity of its alcoholic solution which when added to 1 cc of nutrient broth inhibited completely the growth of the Staphylococcus for 2 days. Suitable controls for the alcoholic content of the extracts were included.

Results. Our Tillandsia extracts, prepared as described above, contain from 400 to 1600 units per cc. Other organisms which had the same order of sensitivity to our extract as the Staphylococcus were Pneumococcus I, II, and III, *Streptococcus hemolyticus* C 203, and *Cryptococcus hominis*. *H. influenzæ* Type B and *Candida albicans* were less sensitive. In these latter experiments, appropriate media for the growth of the organisms and for dilution in the inhibition tests were utilized. Our extract in 1 to 50 dilution (containing the highest concentration of alcohol permissible) had no inhibitory effect on the growth of *H. influenzæ* Type A, *Ps. pyocyaneus* (2 strains), *E. coli* (2 strains), and *B. proteus*.

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[1] Pratt, R., *Science,* 1944, **99**, 351.

[2] Osborn, E. M., *Brit. J. Exp. Path.*, 1943, **24**, 227.

[3] Lucas, E. H., and Lewis, R. W., *Science,* 1944, **100**, 597.

[4] Southam, C. M., and Ehrlich, J., *Phytopathology,* 1943, **33**, 517.

[5] McKee, C. M., Rake, G., and Menzel, A. E. O., *J. Immunol.*, 1944, **48**, 259.

Summary. A method for extracting an antibiotic substance from *Tillandsia usneoides* (Spanish Moss) is described. The crude extract is effective against all the gram-positive bacteria tested and against *Cryptococcus hominis*.

14973

Antifungal Properties of Clavacin.*†

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The value of certain antibiotic substances, such as penicillin and tyrothricin, for the treatment of many bacterial infections is well established, although the vast majority of such agents are considered too toxic for therapeutic use. It is of especial interest to note, however, that an agent such as tyrothricin, which is toxic on parenteral injection, is non-toxic when given orally or applied externally. Thus, in the treatment of certain types of local infections, tyrothricin has met with remarkable success.[1]

The majority of fungous infections are both local and superficial in character. It follows, therefore, that toxicity upon injection does not necessarily disqualify a possible therapeutic agent. Since many fungi are known to be susceptible to the action of antibiotic substances, and since there is a paucity of satisfactory therapeutic agents for the control of mycoses, it appears that the effects of antibiotic agents on the pathogenic fungi should be investigated.

The following report deals with the effect of clavacin on 3 representative species of fungi, viz., *Monilia albicans, Oidium asteroides,* and *Trichophyton gypseum*.‡

In the early part of this work the clavacin was produced in this laboratory according to the methods of Waksman, Horning, and Spencer,[2] employing a strain of *Aspergillus clavatus* known to produce a high yield of clavacin. During the course of the investigation, crystalline clavacin became available and the tests were repeated using 4 samples of this material obtained from other sources.§

Fungistatic Effects. The ability of clavacin to inhibit the growth of fungi was determined by growing the test organisms in the presence of graded concentrations of this agent. Measured amounts of a liquid medium‖ were placed in flasks and sterilized in the autoclave, after which the portions of medium were restored to volume by the addition of sterile distilled water. To each flask was then added, aseptically, the desired quantity of an aqueous solution of clavacin. The 2% stock solution of this compound was self-sterilizing. After thorough agitation of the mixture of the medium and clavacin solution, 25 ml amounts were aseptically dispensed into sterile flasks.

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* Clavacin is also known as clavatin, clavaformin and patulin.

† These investigations were supported in part by a grant supplied by Parke, Davis and Company, Detroit, Michigan.

[1] Hoogerheide, J. C., *Bot. Rev.*, 1944, **10**, 599.

‡ The culture of *T. gypseum* (granular type) was obtained through the courtesy of Dr. C. W. Emmons, U. S. Public Health Service. The others were taken from the culture collection of this laboratory.

[2] Waksman, S. A., Horning, E. S., and Spencer, E. L., *J. Bact.*, 1943, **45**, 233.

§ One sample of crystalline clavacin was kindly supplied by Parke, Davis and Company and 3 samples by the Mallinckrodt Chemical Works, St. Louis, Mo.

‖ Constituents: $NaNO_3$ 2.0 g, KH_2PO_4 1.0 g, KCl 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $MnSO_4 \cdot 4H_2O$ 0.01 g, glucose 20.0 g; peptone 5.0 g, and distilled water to make 1 liter.

Heavy suspensions of each of the test organisms were prepared by washing agar slant cultures with 5 ml amounts of sterile water. One drop of the resulting suspensions was used as an inoculum. After 4 to 7 days of incubation at room temperature, the relative amounts of growth were estimated. The tests were repeated at least twice, using each of the 5 above-mentioned samples of clavacin. It was shown that concentrations of 0.04% and 0.02% clavacin had only a slight inhibitory effect on the growth of *M. albicans* and *O. asteroides*. More dilute solutions produced no visible effect on either of these two organisms. The growth of *T. gypseum,* in contrast, was completely inhibited by these concentrations of clavacin. In the presence of 0.01% of the agent, the response varied from no growth, in the case of two samples, to slight growth in the case of the other samples. A slight, but visible, inhibition occurred in a medium containing only 0.001% clavacin.

Fungicidal Effects. To determine the fungicidal effect of clavacin, the test organisms were exposed to several concentrations of the substance for various periods of time, transferred to a liquid medium and incubated to determine their viability.

Suspensions of the organisms[¶] were prepared as before, filtered through glass wool, centrifuged and the supernatant fluids decanted. The sedimented cells were then suspended in previously prepared aqueous solutions of clavacin. One loopful of each suspension was transferred to 25 ml of a liquid medium[**] at intervals of 15 minutes during the first hour, then at hourly intervals for the next seven hours, and finally after 24 hours. This method is essentially that proposed by McCrea.[††][3]

Two, or in some cases 3, experiments were performed testing each of the 5 samples of clavacin against each of the 3 fungi. There was no evidence of variation in the fungicidal power of the several samples. The 1.0% and the 0.5% solutions were found to be fungicidal for all of the organisms. A solution containing 0.1% clavacin did not kill suspensions of *M. albicans* and *O. asteroides,* even after 24 hours, but was lethal to spores of *T. gypseum* in 2 to 5 hours. For the sake of clarity, the shortest periods of exposure found to be fungicidal are presented in Table I. Inasmuch as 15 minutes was the minimum time interval used, the time involved in the killing action may actually have been less where this figure is given.

TABLE I.
Fungicidal Action of Clavacin on *M. albicans, O. asteroides* and *T. gypseum.*

	Concentration of clavacin		
Test organism	1.0%	0.5%	0.1%
M. albicans	½[*]	¾-3[†]	no effect in 24 hr
O. asteroides	¼-1	1-3	no effect in 24 hr
T. gypseum	¼	¼-¾	2-5

[*] Minimum time of exposure, in hours, found to be fungicidal.

[†] Two figures represent the range as observed in the several experiments.

Summary. Using 3 species of fungi, the fungistatic and fungicidal actions of clavacin were investigated. The presence of 0.02%, and with some samples 0.01%, of clavacin in the culture medium completely inhibited the growth of *Trichophyton gypseum.* A concentration of 0.001% of clavacin caused partial inhibition while 0.0001% caused no detectable effect. In contrast, the presence of 0.04% of clavacin had but a slight inhibitory effect on *Monilia albicans* and *Oidium asteroides.*

A 1% solution of clavacin was fungicidal for *T. gypseum* in 15 minutes, the shortest exposure time employed, *M. albicans* in 30 minutes, and *O. asteroides* in 15 minutes to 1 hour. Solutions containing 0.5% clavacin were fungicidal for *T. gypseum* in 15 to 45 minutes, to *M. albicans* in 45 minutes to 3 hours, and for *O. asteroides* in 1 to 3 hours. The 0.1% solutions killed *T. gypseum* in 2 to 5 hours but failed to be fungicidal for the other two organisms even after 24 hours.

[¶] Vegetative cells of *M. albicans;* oidia of *O. asteroides;* principally microconidia of *T. gypseum.*

[**] See footnote ||.

[††] In an earlier article (Herrick, J. A., and Kempf, J. E., *J. Bact.*, 1944, **48**, 331) attention was directed to the large amount of sodium azide transferred with the inoculum and the consequent unsuitability of the procedure in that study.

[3] McCrea, A., *J. Lab. Clin. Med.*, 1931, **17**, 72.

14974 P

Characteristics of the Mammary Tumor Milk Agent in Serial Dilution and Blood Studies.*

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The mammary tumor milk agent has been shown to be essential for the regular development of mammary cancer in mice.[1] It is usually transferred by nursing[1] and is present in various tissues[3-6] as well as the milk. The infectious agent has the characteristics of a virus.[7]

This report gives data on the activity of the milk agent for mammary cancer in blood plasma, blood-cell suspensions, and serial dilution of extracts made from mammary tissue.

Blood was obtained from the hearts of mice known to harbor the agent. Either females of the A stock, 75 to 105 days of age, or females (A and C3H stocks and their hybrids) that had developed spontaneous mammary cancer were used. The blood was citrated to prevent coagulation but moderate hemolysis occurred. Separation of the cells and the plasma was by centrifugation (approximately 4,000 r.p.m. for 10 minutes) and the cells were washed with saline several times before they were used. The cells were suspended in saline and the plasma was injected either undiluted or diluted in saline (see table for details).

As test animals mice were used that were susceptible for mammary cancer and lacking the milk agent. They were injected intraperitoneally when from 3 to 5 weeks of age. The incidence in control mice maintained as breeding females was approximately 1%.

The results for the blood studies are presented in Table I. Many of the mice in one series are living at 15 months of age.

The donors of the lactating mammary tissue were AZF_1 females that had had from 5 to 7 litters. The glands were macerated by means of a press, and serial dilutions were made with triple distilled water. Each animal received 1 cc of the suspension intraperitoneally and the surviving animals are 15 months of age. The preliminary results are tabulated in Table II.

Discussion. Woolley *et al.*[6] determined that the milk agent of mammary cancer could be obtained by the use of whole blood. The low incidence (19%) obtained in the experimental animals might have been influenced by the age of the test animals at the time of injection.[7,8]

When blood was obtained from young donors 75 to 105 days of age, the results, based on small numbers, suggested that the agent might be either contained within or adsorbed on the blood cells.[5] These results could not be confirmed in preliminary data secured after the testing of cell-suspension and plasma from mice that had developed spontaneous mammary cancer. In other experiments in which the mice received the agent by nursing, the data suggested that the agent might be more concentrated in older mice.[7] Whether or not the differences observed in the studies on the blood components may be explained on the same basis must wait further investigations.

If the amount of the milk agent obtained by

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* Assisted by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the Graduate School Cancer Research Fund, University of Minnesota.

[1] Bittner, J. J., *Science*, 1936, **84**, 162.

[2] Bittner, J. J., *Cancer Research*, 1942, **2**, 710.

[3] Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 805.

[4] Bittner, J. J., *Bull. Minn. Med. Found.*, 1944, **4**, 94.

[5] Bittner, J. J., Evans, C. A., and Green, R. G., *Science*, 1945, **101**, 95.

[6] Woolley, G. W., Law, L. W., and Little, C. C., *Cancer Research*, 1941, **1**, 955.

[7] Bittner, J. J., *Cancer Research*, 1942, **2**, 540.

[8] Andervont, H. B., Shimkin, M. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1942, **3**, 309.

TABLE I.

Material injected	Dilution	Amount, cc	No.	% cancerous
Plasma*	0	1	15	7
Cell-suspension*	1:2	1	10	60
Plasma†	1:2	1	10	50
Plasma†	1:2	0.5	11	36
Cell-suspension†	1:2	1	22	18
Cell-suspension†	1:2	0.5	13	46

† Donors—mice with spontaneous mammary cancer, preliminary data.
* Donors 75 to 105 days of age.

TABLE II.

Dilution	No. injected	% cancerous	No.	% cancerous
1:5000	22	41	41	46
1:1000	19	53		
1:100	18	28	18	28
1:10	19	11	50	8
1:5	31	7		

nursing influences the incidence and/or average cancer age, this may not be the case in older mice that receive injected material.

When mice 3-5 weeks of age were injected intraperitoneally with extracts of mammary tissue containing the milk agent, more mammary tumors have been observed in the mice that received the more dilute suspensions. Although many of the animals are still under observation, the data indicate that further work is essential to account for the results. There are several possibilities: (a) the presence of an inhibitory agent; (b) destructive effect by autolysing enzymes; (c) the development of active immunity with the injection of massive doses; (d) genetic differences between hosts and donors; etc.

14975

Dental Caries in the Cotton Rat. V. Influence of Strain Variation on the Caries Susceptibility.*

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The effect of diet on the extent of tooth decay in the cotton rat has been studied in a series of investigations.[1,2,3,4] Considerable variation was observed in the incidence and

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[1] Shaw, J. H., Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Phillips, P. H., *J. Nutrition*, 1944, **28**, 333.

[2] Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dental Res.*, 1944, **23**, 417.

[3] Schweigert, B. S., Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutrition*, in press.

[4] Shaw, J. H., Schweigert, B. S., Phillips, P. H., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., in press.

TABLE I.
Effect of Strain Variation on Incidence and Extent of Carious Lesions.

Parent stock No.	Cariogenic diets							Non cariogenic diets				
	No. of animals	No. of carious lesions		Analysis of variance (F value)	Extent of carious lesions		Analysis of variance (F value)	No. of animals	No. of carious lesions		Extent of carious lesions	
		Range	Avg		Range	Avg			Range	Avg	Range	Avg
2	13	16-39	28.7	2.0	21-148+	89+	1.4	7	0-16	6.7	0-33+	16+
5	18	22-37	31.3		63-131+	100+		—	—	—	—	—
6	16	25-39	32.4	.5	53-153+	102+	.1	3	8-17	11.3	9-22+	16+
7	13	12-34	19.9	36.9*	18-110+	51+	31.8*	2	0- 2	1.0	0- 5+	3+
9	3	30-34	32.3		86-126+	101+		2	1- 5	3.0	5-10+	8+
10	3	33-35	34.0		86- 99+	94+		3	0-13	6.3	0-18+	11+
11	7	5-23	13.0	71.0*	13- 60+	30+	84.6*	—	—	—	—	—
12	13	0-33	19.8	24.3*	0-109+	55+	28.4*	1	0		0	
13a	2	29-31	30.0		106-120+	113+		1	0		0	
14	11	28-39	33.7	2.43	77-156+	113+	2.37					
16	5	22-37	28.2		60-116+	89+						
16a	3	26-40	31.0		78-158+	113+						
17 (12♂, 14♀)	3	15-37	26.7		29-124+	80+		—	—	—	—	—
18 (12♂, 12♀)	2	14-20	17.0		34- 39+	37+						
19 (12♂, 12♀)	3	5-16	11.7		10- 48+	33+						
101	2	21-27	24		63- 83+	73+		3	0		0	
103	3	22-30	24.7		38-102+	63+		3	0		0	
X	6	10-19	14.1		17- 41+	29+		5	0- 3	1.2	0- 4+	2+
200	3	19-28	24.3		33- 83+	66+		3	0-14	4.7	0-22+	7+
203	4	14-27	18.3		23- 83+	44+		4	0- 3	1.0	0-11+	3+

* Highly significant as compared to No. 5. The 1% values for F ranged from 7.60 to 7.88 for the groups statistically analyzed as obtained from *Statistical Tables for the Biological, Agricultural and Medical Research* by R. A. Fisher and F. Yates (Oliver and Boyd, 1938).

extent of carious lesions in offspring from different parents. A study of these variations is presented in this paper.

Experimental. The incidence and extent of carious lesions were observed in rats when fed diets containing soluble sugars and when fed dextrin or stock rations by the procedures outlined in earlier work.[1,2] The former rations are referred to collectively as cariogenic and the latter as non-cariogenic. Pairs of stock animals designated from 2-19 inclusive are from our stock colony, and pairs 101-103 were purchased as adults from Florida.[†] Young animals (30-50 g in weight) were obtained from Michigan.[‡] Six of the latter animals were fed the sucrose basal diet[1] and 5 were fed a stock ration for 14 weeks. These groups have been designated X. The remainder of the animals from Michigan were used for the stock colony (pairs 200-205). Offspring from these animals were used to test their susceptibility to caries as described in earlier work.[1,3] The incidence and extent of the carious lesions in offspring from each pair of parent stock from the Wisconsin, Florida, and Michigan strains are presented in Table I. A statistical analysis of the data for offspring from our stock colony is also presented. The incidence and extent of caries in each case are compared to those of the offspring from parents No. 5. Only those groups where 7 or more animals are represented have been statistically analyzed.

Results. It is evident from the data that offspring from different parents as well as animals from the same parents show a wide variation in caries susceptibility. It should be pointed out that the offspring represented in the Wisconsin strains (Nos. 2-14) are representatives of litters obtained over a period of 16 months. This long period may increase the variation within the total number of brother-sisters tabulated. Within the Wisconsin strains, offspring from parents No. 2, 5, 6, 9, 10, 13a, 14, 16, 16a, and 17 have a much higher average incidence and extent than those from parents 7, 11, 12, 18, and 19. Within any experimental series, animals from the latter stock have a lower incidence and extent than those of the former. The differences between the offspring from No. 5 and those from No. 7, 11, or 12 are highly significant. The small differences observed when offspring from No. 5 are compared to 2, 6, or 14 are not significant by statistical analysis. An extremely low incidence is observed when the non-cariogenic diets were fed as compared to the caries index of the brother-sister animals receiving the cariogenic diets. Offspring of the less susceptible strains can be used for experimental comparisons since an adequate difference in the incidence and extent of tooth decay has been observed between the animals fed the cariogenic and non-cariogenic diets.

It has been observed that offspring from parents No. 12 have been the most variable in the degree of tooth damage. In fact one offspring from this pair was devoid of caries and another animal from a subsequent litter had 33 cavities at the end of the 14-week experimental period. These variations show the necessity of adequate distribution of animals from various parents within a given experimental series as has been pointed out earlier.[3] Variations between siblings of one litter from one pair of breeding stock are much less than for all the siblings of all litters from that pair.

Second generation offspring from No. 12 (No. 18 and 19) also show low susceptibility to dental caries. The amount of tooth damage obtained is similar to that in siblings of the parents. Thus it appears that susceptibility is influenced by inheritance. Matings made subsequent to these observations will further elucidate the effect of inbreeding and of crossing susceptible and resistant strains on the incidence and extent of tooth decay.

The offspring of the Michigan and Florida stocks show a lower susceptibility to dental caries as compared to the susceptible Wisconsin stock and approximate the more resistant Wisconsin stock in the degree of susceptibility.

[†] Purchased from the Hegener Research Supply Company, Box 224, Indian Beach, Sarasota, Florida.

[‡] Obtained from Michigan State Board of Public Health.

Discussion. Hunt and co-workers[5] have studied the inheritance of susceptibility to dental caries in the white rat, and have obtained in a series of 12 generations, a marked increase in susceptibility by inbreeding susceptible strains. They could decrease the susceptibility of the resistant strains by similar means. McClure and Arnold[6] in a study on the influence of fluorides and iodoacetate on the incidence of caries in the white rat have pointed out a difference in caries susceptibility of offspring from different parent stock. Arnold[7] has also observed that a difference in caries susceptibility existed in hamsters, when offspring from different parents were compared. These observations indicate the importance of heredity as a factor in the production of experimental dental caries and also point out the necessity of recognizing this factor in designing properly controlled experiments, especially where animals are of heterogeneous stock.

Summary. A difference in susceptibility of different strains of cotton rats to dental caries has been observed and shown to be statistically significant. Preliminary results from second generation offspring indicate that their susceptibility is similar to that of the parent stock.

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[5] Hunt, H. R., Hoppert, C. A., and Erwin, W. G., *J. Dental Res.*, 1944, **23**, 385.

[6] McClure, F. J., and Arnold, F. A., Jr., *J. Dental Res.*, 1941, **20**, 97.

[7] Arnold, F. A., Jr., *U. S. Pub. Health Rep.*, 1942, **57**, 1599.

14976

Effect of Cardiac Cycle Length upon Magnitude of Ventricular Gradient.

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The ventricular gradient of Wilson[1] may be defined as the net electrical effect of differences in time course of repolarization of different parts of the ventricular muscle as this effect is projected onto the frontal plane of the body. In the electrocardiogram it is measured by the mean manifest net area of the QRS-T group of deflections. In previous papers[2,3] it has been pointed out that the magnitude of the gradient varies inversely as the heart rate or directly as the average cardiac cycle length. The present report seeks to explain this relationship.

Three main procedures were used. In one, the string galvanometer recorded the action current derived from the apex and base of the excised turtle ventricle, in air. Apical negativity yielded an upward deflection. Records were obtained when the ventricle was driven at various rates, as shown by the cycle lengths in Table I. In a second set of experiments the apex was cooled, though, as the irregularity of the results shows, no attempt was made to keep the temperature constant. Table I also shows the Q-T interval for each cycle length, and the net area of QRS-T in units of about 30 microvolt-seconds. The method of measuring the areas has been published elsewhere.[4] Since the initial deflections were small, the larger part of the net QRS-T area is within the T wave, except when the cycles were short.

It may be observed that the net areas are

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[1] Wilson, F. N., Macleod, A. G., and Barker, P. S., *Tr. A. Am. Physicians*, 1931, **46**, 29.

[2] Ashman, R., Ferguson, F. P., Gremillion, A. I., and Byer, E., *Am. J. Physiol.*, 1945, **143**, 453.

[3] Ashman, R., and Byer, E., *Am. Heart J.*, 1943, **25**, 36.

[4] Ashman, R., Byer, E., and Bayley, R. H., *Am. Heart J.*, 1943, **25**, 16.

TABLE I.
Relationship between Cycle Length and Net QRS-T Areas.

Cycle lengths (sec)	Apex not cooled: Net QRS-T areas	Apex not cooled: Q-T intervals (sec)	Apex cooled: Net QRS-T areas	Apex cooled: Q-T intervals (sec)
20	—220	1.70	+268	1.92
10	—200	1.66	+380	2.12
7	—136	1.60	+280	2.10
5	— 76	1.52	+198	2.02
2	— 20	1.30	— 4	1.42
1.2	— 8	0.84	— 52	0.88

TABLE II.
Relationship between Cycle Length and Duration of Monophasic Curve.

Cycle lengths (sec)	Ventricle at room temp. (24°C) Duration of monophasic curve (sec)	Ventricle 11.5°C below room temp. Duration of monophasic curve (sec)	Ventricle 20°-18°C below room temp. Duration of monophasic curve (sec)
20	1.40	2.70	4.40
10	1.40	2.65	4.00
7.5	1.40	2.47	2.96
5	1.40	2.27	2.35
4.2	untested	untested	1.87
4	”	”	1.67
3	”	1.65	refractory
2	”	1.10	”
1.4	1.05	refractory	”
1.1	0.77	”	”

negative, that is, below the base line of the electrogram, when the apex was at the room temperature of 24°C. This finding indicates that in this heart the repolarization of the apex was more rapid than that of the base. The significant finding is the very small area when the cycles were shortest, and the progressive decrease in area as the minute heart rate increased. This was the consistent result in 5 experiments.

Table I also shows that the Q-T interval decreased progressively as the cycle length was shortened. In major part this was due to the well known effect of cycle length change upon Q-T duration; in part, in this case, it was a cumulative effect of activity.[5]

When the apex was cooled, the time required for repolarization in that region was longer than it was at the base. This is demonstrated by the increase which occurred in the Q-T interval. The cooling was not quite so great at the 20-second cycle as it was throughout the rest of the experiment. At the longer cycle lengths the net QRS-T areas were positive, but at the very short cycles, the net areas became negative, in spite of the fact, shown by a control curve at the end of the experiment, that the apex was still cool. It is noteworthy that in this experiment, when the cycles were very short, the base became repolarized more slowly than the apex. This means that the inherent tendency of the base to recover more slowly than the apex in this heart again manifests itself when the increased rate greatly shortened the recovery time at the cool apex. This latter phenomenon is often seen in the human heart when, as a result of a locally deficient blood supply, a part of the ventricular muscle becomes repolarized more slowly than the rest. Then, in association with a premature beat, a normal form of the T wave is often restored. The ischemic muscle evidently behaves like cool muscle in this respect.

The third procedure reveals the cause of these changes. The ventricle was immersed in Ringer's solution at room temperature

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[5] Blair, H. A., Wedd, A. M., and Young, A. C., *Am. J. Physiol.*, 1941, **132**, 157.

(24°C) and again in a cooler solution. By means of a suction electrode[6] injury was produced, and the monophasic curves were recorded when the galvanometer was connected to this electrode and to another electrode several centimeters distant in the solution. Table II shows that when the cycles were long the monophasic curve of the cool muscle, measured from the top of its ascending limb to its end, had nearly double the duration of the curve derived from the uncooled ventricle. When the cycles were short, however, the monophasic curves were practically equal in duration at both temperatures. This experiment was typical of four. It explains the effect of rate increase in the uninjured heart, namely, that the decrease in the net QRS-T area is due to a change in the relative times required for repolarization, in the sense that the time becomes more nearly the same in all parts of the muscle.

This evidence indicates that in the human heart, beating slowly, the large gradient which is usually present (and which accounts for the normal T-wave directions) is due to the slower repolarization of some parts of the muscle than of other parts. When the rate is very rapid, the time course of repolarization is everywhere nearly the same, and the gradient is, therefore, reduced in magnitude.

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[6] Wiggers, H. C., *Am. J. Physiol.*, 1938, **123**, 215.

14977

Form Variation in Group C Salmonella Strains.*†

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The occurrence of strains of the Newport group (VI, VIII) which lacked antigen VI, the O component possessed in common with the Cholerӕ-suis (VI, VII) and Carrau-Onderstepoort (VI, XIV . . .) groups, occasioned a closer study of the O antigens of group C Salmonella cultures. Kauffmann[1] demonstrated that antigen VI is divisible into at least 2 fractions which he designated VI_1 and VI_2. The majority of cultures of *S. cholerӕ-suis* contained only VI_1 antigen, although a few contained only VI_2. The remaining strains of the VI, VII group which were studied contained both VI_1 and VI_2. On the contrary, cultures of the Newport group (VI, VIII) contained only VI_1. Hormaeche, Peluffo, and Ricaud de Pereyra[2] found that antigen VI was more complex than had been generally realized and that it was composed of at least 3 principal fractions and additional minor antigens. Further, they remarked that they had found its distribution rather irregular. Also, in routine typing it has been observed that cross agglutination between the VI, VII and VI, VIII groups was quite variable. The lack of antigen VI in certain cultures and the irregularities noted in its behavior suggested that the antigen, or a portion thereof, was subject to the form variation or O variation which Kauffmann[3,4] found in antigen I and in a fraction of antigen XII. Accordingly, cultures of *S. newport* and *S.*

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* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

† The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Kentucky Agricultural Experiment Station.

[1] Kauffmann, F., *Z. f. Hyg.*, 1937, **120**, 177.

[2] Hormaeche, E., Peluffo, C. A., and Ricaud de Pereyra, V., *J. Bact.*, 1944, **47**, 323.

[3] Kauffmann, F., *Acta path. et microbiol. Scand.*, 1940, **17**, 135.

[4] Kauffmann, F., *J. Bact.*, 1940, **41**, 127.

TABLE I.
Form Variation in VI, VIII Types.

Antigens	Serums VI$_1$, VI$_2$, VIII 20	40	100	200	400	VI$_1$, VIII 100	200	400	1000	2000	I, VI, XIV, XXV 20	40	100	200	400
Newport 563, colony 14 (±)	±	—	—	—	—	++++	+++	+++	++	—	±	±	±		—
Newport 563, colony 21 (++)	++++	+++	+++	+++	+	++++	+++	+++	++	—	++++	++++	++++	++++	++
Oregon 1511, colony 2 (±)	±	±	—	—	—	++++	+++	+++	++	—	±	±	±	—	—
Oregon 1511, colony 9 (++)	++++	+++	+++	+++	+	++++	+++	+++	+	—	++++	++++	++++	+++	+

oregon, the O antigens of which are delineated as VI$_1$, VIII, were plated and individual colonies examined by slide agglutination with VI$_1$,VI$_2$,VII and VI$_1$,VIII serums. The latter serum was used only to check the agglutinability of the individual colonies and all were agglutinated equally well by it. The behavior of the colonies in VI$_1$,VI$_2$,VII serum divided them into 2 groups. Some colonies were agglutinated quickly and completely (++) while in others there was only a trace of agglutination (±) after several minutes. In the particular strains examined the numbers of VI± and VI++ colonies were nearly equal. The remains of the colonies were picked to agar slants and alcohol-treated antigens prepared by the method of White.[5] Tube tests yielded the results given in Table I. It is evident that in *S. newport* and *S. oregon* the content of antigen VI$_1$ is quite variable. In order to ascertain the stability of the two forms, plates were streaked from the agar slant cultures which had been used to prepare the alcohol-treated antigens. Two cultures of each form of *S. newport* and *S. oregon* were plated. Twenty colonies on each plate were tested and it was found that 5 to 20% of the colonies had reverted to the opposite form. Thus it may be seen that *S. newport* and *S. oregon* contain VI± and VI++ forms and that each form reverts naturally to the other. The same type of variation was found in a non-motile VI,VIII culture received from Major K. S. Wilcox.

After the above results were obtained, *S. hartford, S. mikawashima, S. oranienburg,* and *S. montevideo* of the VI$_1$,VI$_2$,VII group were examined in a similar manner. Colonies were tested with VI$_1$,VIII and VI$_1$,VI$_2$,VII serums. Again it was found that VI± and VI++ forms were recognizable. The results obtained with representative single colony cultures are given in Table II. These forms also were found to revert to the opposite form under ordinary conditions of culture. Absorptions were done with a number of VI± forms derived both from VI, VII, and VI,VIII cultures. In general it may be said that absorption of a VI,VII serum with a VI±

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[5] White, P. B., Med. Res. Counc. Gt. Brit., Spec. Rpt. Ser. No. 103, 1936.

TABLE II.
Form Variation in VI, VII Types.

Antigens	Serums														
	VI_1, VIII					VI_1, VI_2, VII					I, VI, XIV, XXV				
	20	40	100	200	400	100	200	400	1000	2000	20	40	100	200	400
Hartford 3015, colony 9 (±)	++	+	±	−	−	++++	+++	+++	+	−	++++	++++	+++	++	+
Hartford 3015, colony 16 (++)	++++	+++	+++	+	±	++++	+++	+++	±	−	++++	++++	++	+	±
Montevideo 623, colony 1 (±)	+	±	−	−	−	++++	+++	+++	+	−	++++	++++	+++	+	±
Montevideo 623, colony 9 (++)	+++	+++	+++	−	−	++++	+++	+++	+	−	++++	++++	+++	+	±
Mikawashima 45, colony 4 (±)	+	±	−	−	−	++++	+++	+++	+	−	++++	++++	++++	+++	+
Mikawashima 45, colony 7 (++)	+++	+++	+++	±	−	++++	++++	+++	+	−	++++	++++	++	+	±
Oranienburg 9945, colony 1 (±)	+	±	−	−	−	++++	++++	++++	+	−	++++	++++	++++	+++	++
Oranienburg 9945, colony 7 (++)	++++	+++	+++	+	−	++++	++++	+++	+	−	++++	++++	++++	++	+

form derived from a VI,VIII culture removed all agglutinins for itself and other similar forms but left traces of agglutinins for all VI++ forms derived from VI,VIII cultures. Considering the amount of reversion that occurs in the VI± forms, one would not expect a large residue of VI_1 agglutinins to be left in the serum. Similar absorption tests with VI± forms derived from VI,VII cultures in which VI,VIII serum was used gave comparable results.

It should be emphasized that the results outlined above apply only to that portion of antigen VI which has been designated as VI_1. The VI,VIII cultures contained only VI_1 and the VI,VIII serums used to test the VI,VII strains contained only agglutinins for VI_1. While it is not intended to enter into an extended analysis of antigen VI, it is desirable to know whether the variation described affects only VI_1 or the whole VI complex. This was easily determined by testing ± and ++ forms of VI,VII and VI,VIII cultures with serum derived from *S. onderstepoort* (I, VI, XIV, XXV) which contains agglutinins both for fractions VI_1 and VI_2. These tests are included in Tables I and II. It is evident that the ± and ++ components of the VI,VIII cultures, which contain only VI_1, react in *S. onderstepoort* serum as they do in VI,VII serum. On the contrary both the VI± and VI++ forms of the VI,VII types, which contain both VI_1 and VI_2, react strongly in *S. onderstepoort* serum. It may be said, therefore, that a colony which contains VI_1 and VI_2 antigens may possess only a ± complement of VI_1 but still have a full complement of VI_2. Further, 100 colonies of a culture of *S. choleræ-suis* var. *kunzendorf,* the O antigens of which were VI_2, VII, were tested with *S. onderstepoort* serum. All the colonies reacted equally well and there was no indication that any O variation occurred. While the results with one strain are not conclusive, they strongly suggest that VI_2 is not variable. Certainly variation in VI_1 and VI_2 does not go hand in hand.

Certain cultures of group C seem to be totally and permanently lacking in antigen VI. Among such cultures are *S. amherstiana* (VIII:1,v-1,6 . . .) of Edwards and Bruner[6] and the recently described *S. virginia* (VIII:d) of Saphra and Seligmann.[7] In addition a culture received from the Virginia State Department of Health had the formula VIII:d-1,2,3 . . . Also two cultures of *S. newport* isolated from a snake by Dr. W. R. Hinshaw had only antigen VIII. Non-motile variants isolated from the same animal had identical O antigens. It is well known that antigens that are subject to form variation may be totally lacking in certain cultures. For instance, Kauffmann demonstrated variation involving antigen I in a number of types of groups A and D. Cultures of these types are found which contain no I antigen. Likewise Kauffmann found that a fraction of antigen XII (XII$_2$) was subject to variation and that it was totally lacking in a strain of *S. typhi*, a species which ordinarily contains the antigen. Therefore, it is to be expected that VI$_1$ would be lacking in certain VI$_1$,VIII strains. These probably represent loss variants of cultures with typical O antigens.

Summary. It was found that antigen VI$_1$ was subject to a reversible form variation similar to that reported by Kauffmann for antigens I and XII$_2$. This variation occurred both in the *S. choleræ-suis* and *S. newport* groups. Colonies which were deficient in VI$_1$ had a normal content of VI$_2$. So far as is known the latter antigen is not subject to form variation.

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[6] Edwards, P. R., and Bruner, D. W., *J. Immunol.*, 1942, **44**, 319.

[7] Saphra, I., and Seligmann, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 50.

14978

A new Salmonella type: *Salmonella pensacola.*[*][†]

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Salmonella pensacola, the type to be described, was isolated from a severe case of acute gastro-enteritis in a laboratory worker and was forwarded to the writers by Mrs. Mildred Galton. The source of infection was not known. On preliminary examination, the culture proved to be a motile rod which possessed the usual cultural and biochemical characteristics of the Salmonella group. Hydrogen sulfide was produced and Jordan's tartrate was fermented but indol was not formed nor was gelatin liquefied. Acid and gas were formed in 24 hours from glucose, rhamnose, xylose, arabinose, maltose, trehalose. dulcitol, mannitol, sorbitol, and inositol. Cellobiose was fermented after 6 days incubation. Lactose, sucrose, raffinose, and salicin were not attacked. Dextro-tartrate, levo-tartrate, mucate, and citrate were utilized but meso-tartrate was not attacked.

On serological examination the organism was found to be a member of group D of the Kauffmann-White classification and in absorption tests it completely removed agglutinins from *S. gallinarum* serum. The O antigens of *S. pensacola* are IX, XII. When the H antigens were examined it was found that *S. pensacola* was flocculated in low dilution by the majority of serums derived from forms related to *S. enteritidis*, *i.e.*, the monophasic forms containing antigen g. However it was agglutinated in high dilution only by serums derived from *S. california* (IV,XII:g,m,t . . .) and *S. oranienburg* (VI,VII:m,t . . .) In these two serums it was agglutinated to titer. When

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[*] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

[†] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Kentucky Agricultural Experiment Station.

TABLE I.
Agglutinin Absorption Tests × 100.

	Serums														
	Oranienburg (m,t . . .)			California (g,m,t . . .)				Pensacola						
		Absorbed by			Absorbed by				Absorbed by					
Antigens	Unabsorbed	Pensacola	California (g,m,t . . .) + Pensacola	Unabsorbed	Pensacola	Oranienburg (m,t . . .) + Enteritidis (g,m . . .) + Pensacola	Derby (f,g . . .) + Pensacola + Oranienburg (m,t . . .) + Enteritidis (g,m . . .)	Unabsorbed	Oranienburg (m,t . . .)	California (g,m,t . . .)	Berta (f,g,t . . .)	Budapest (g,t . . .)	Oranienburg (m,t . . .) + Berta (f,g,t . . .) + Moscow (g,q . . .)
Derby (f,g . . .)	<1	<1	<1	5	5	2	<1	2	1	1	<1	<1	<1
Berta (f,g,t . . .)	10	<1	<1	20	5	<1		100	1	1	<1	10	<1
Enteritidis (g,m . . .)	2	2	<1	10	10	<1	<1	2	<1	<1	1	1	<1
Montevideo (g,m,s . . .)	5	2	1	1	<1	<1		5	<1	2	5	5	<1
California (g,m,t . . .)	50	10	<1	400	100	2	<1	100	1	<1	20	10	<1
Dublin (g,p . . .)	<1	<1	<1	10	10	<1		5	<1	<1	2	2	<1
Rostock (g,p,u . . .)	<1	<1	<1	10	10	<1		1	<1	<1	<1	<1	<1
Moscow (g,q . . .)	<1	<1	<1	10	<1	<1		20	5	2	1	1	<1
Senftenberg (g,s,t . . .)	2	<1	<1	10	<1	<1		10	<1	<1	10	<1	<1
Budapest (g,t . . .)	5	<1	<1	20	1	<1		50	<1	1	20	<1	<1
Oranienburg (m,t . . .)	50	20	5	400	50	<1	<1	100	<1	2	100	10	<1
Pensacola	50	<1	<1	400	<1	<1	<1	400	200	200	200	200	20

Figures indicate highest dilution at which agglutination occurred.

tested in absorbed serums for single factors f, m, p, q, s, t, and u it was flocculated vigorously by m and t serums but was unaffected by the remainder. Agglutinating serum was then prepared from *S. pensacola*. Results of pertinent agglutination and agglutinin absorption tests are given in Table I. The tests not only demonstrated the close relationships existing among *S. california, S. oranienburg,* and *S. pensacola* but they also emphasized the complicated mosaic of antigens present in *S. enteritidis* and related forms. It was not surprising that *S. pensacola* possessed a strong specific factor since the complexity of the antigens represented by the symbol t is well known.[1,2,3,4] Inasmuch as *S. pensacola* is very closely related to *S. oranienburg* and *S. california,* is agglutinated by serums for factors m and t and has definite relationships to the remainder of the *S. enteritidis* group, it is proposed to designate the type by the formula IX,XII:g,m,t . . . For diagnostic purposes it is not necessary to introduce a new symbol to designate the specific antigen of the culture and until such time as that becomes necessary it seems undesirable to further complicate the diagnostic schema by the introduction of new symbols.

Summary. A new Salmonella type isolated from gastro-enteritis in man was described. It was called *S. pensacola* and was assigned the formula IX,XII:g,m,t . . .

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[1] Kauffmann, F., *Z. f. Hyg.*, 1937, **120**, 177.

[2] Rauss, K., *Z. f. Immunitatsf.*, 1939, **95**, 489.

[3] Hormaeche, E., Peluffo, C. A., and Salsamendi, R., *Arch. Urug. de Med., Cir. y Espec.*, 1938, **12**, 377.

[4] Edwards, P. R., Bruner, D. W., and Hinshaw, W. R., *J. Inf. Dis.*, 1940, **66**, 127.

14979

Effect of Feeding Calcium Arabonate in Diet of Rats for Successive Generations.

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In former studies with carbohydrates, various sugar alcohols were fed to rats for long periods of time.[1] These substances failed to affect deleteriously the rate of growth in 3 successive generations of rats. Recently calcium arabonate has been suggested as a dietary supplement to provide additional calcium in the diet. As such a use would indicate rather large doses of this compound it was deemed advisable to conduct similar long-term feeding experiments with rats.

Material and Methods. Calcium arabonate is a white crystalline powder, 2% soluble in water at 20°. The compound is obtained readily in a pure form from intermediates used in the synthesis of riboflavin. In the form of its normal salt crystallized with 5 molecules of water calcium arabonate possesses a specific rotation of $[\alpha]_D^{25}$-3.0° in 2% water solution.

Fifteen young male and 15 young female rats were selected for this study. The average weight of each group was 106 g at the beginning of the experiment. Five percent calcium arabonate was mixed intimately with the food of each group of animals. A balanced diet of purina chow fortified with additional vitamins served as the basic diet. On the 24th day one male and one female were placed in each of 15 cages. At periods of time thereafter male animals were sacrificed for histologic examination of the important viscera. This experimental plan was followed with 2nd and 3rd generation rats. A summary of the data obtained is illustrated in the form of growth curves compared with a group of untreated animals from the stock colony of the laboratory (Fig. 1).

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[1] Ellis, F. W., Carr, C. J., Wiegand, E. J., Krantz, J. C., Jr., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 260.

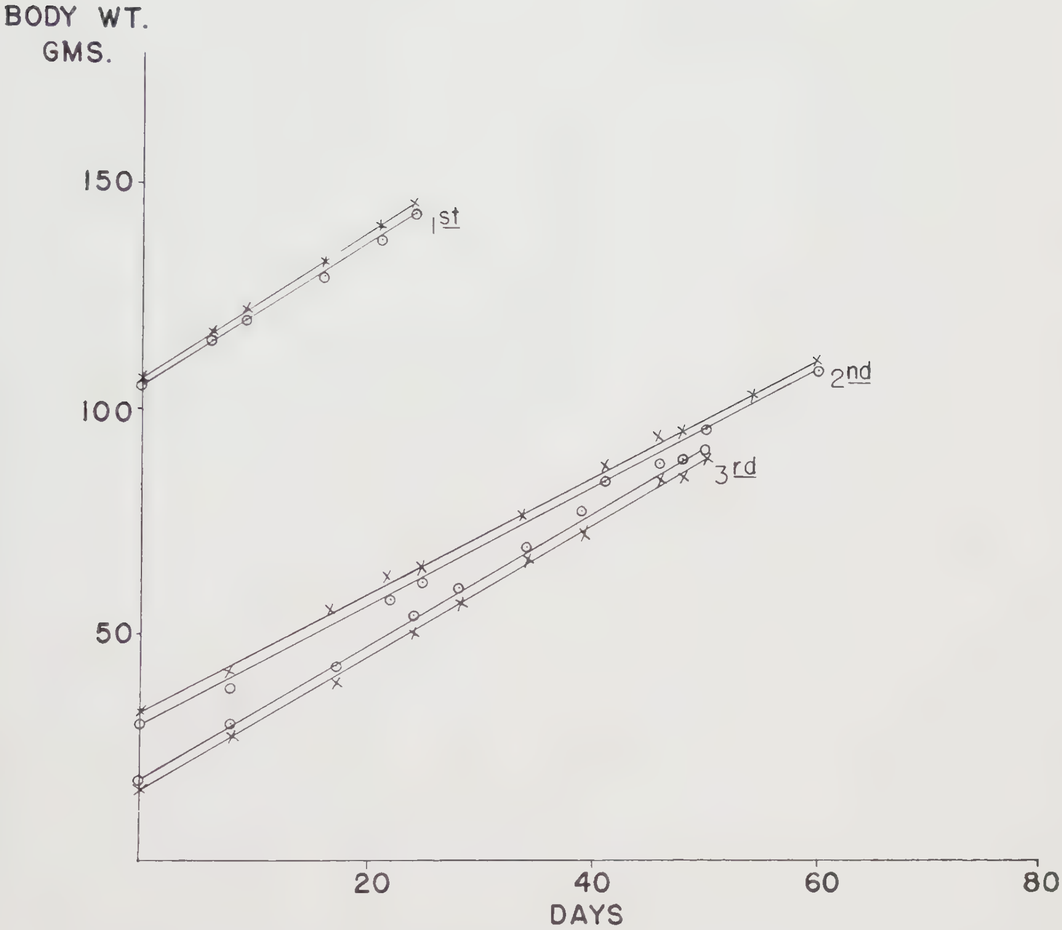

Fig. 1.

Growth curves of first, second and third generation rats fed 5% calcium arabonate. ○ = experimental animals. × = littermate controls. Days fed calcium arabonate.

First Generation Rats. These rats produced 70 young in 50 days. Throughout this period the animals appeared in a good nutritional state. After 74 days breeding was discontinued but the animals continued to receive the calcium salt in their food. At the termination of the experiment these animals were sacrificed and the organs subjected to examination microscopically.

Second Generation Rats. Twenty-four animals were weaned at the age of 21 days and started on the experimental feeding. A similar group comprised of the control animals did not receive the calcium arabonate in their food. At this time the males and females were separated. At the completion of the growth curve experiment 8 pairs of animals were mated. The remainder of the animals continued to receive the calcium salt in their food. All of these rats remained in an excellent nutritional state. From the animals that were mated 84 offspring were produced.

Third Generation Rats. A group of offspring was selected at the age of 21 days and fed the calcium salt in their food. A similar group comprised the control animals. The male and female animals were kept separate and a growth curve was plotted for each group. When these rats had attained a body weight of 100 g the experiment was terminated.

Clotting Time. The influence of feeding calcium arabonate in the food of rats was further studied by determining the clotting time of blood of animals selected at random before and after the experimental period. The clotting time of blood was not significantly altered.

Histologic Studies of Viscera. Twenty-three animals in all were autopsied and sections were prepared from the brain, heart, liver, and kidney for microscopic examination. Grossly no pathologic signs were observed.

Microscopic sections from the first generation animals appeared normal in character. The liver of some animals displayed a granularity of cytoplasm that was thought to be of little significance in connection with these experiments. Two animals in this group each having consumed 107 g of calcium arabonate in their food over a period of 215 days, were autopsied. No pathological lesions were observed grossly or microscopically in these animals. Male and female animals from subsequent generations failed to demonstrate signs of pathologic changes.

Conclusions. The feeding of 5% calcium arabonate in the diet of the white rat for 3 generations does not significantly alter the growth pattern or fecundity. No significant pathological lesions were observed in the brain, heart, liver, or kidney of these animals. The blood clotting time was not influenced.

The calcium arabonate used in this investigation was kindly supplied by the Hoffmann LaRoche Co., Inc., of Nutley, N.J.

14980

Experimental Atherosclerosis. VII. Effect of Vitamin E.

MAURICE BRUGER.

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Dam and Kelman[1] showed that vitamin E exerts an effect on the plasma lipids of the vitamin E-deficient chick similar to that of lipocaic. Since considerable lipotropic activity was ascribed to this vitamin, studies were initiated to determine the effect of alpha-tocopherol on experimental atherosclerosis in rabbits. During the course of these experiments, Dam[2] published his work on cholesterol-fed rabbits and showed that vitamin E, fed as 10 mg% *d,l*-alpha-tocopherol acetate in the basal diet, failed to modify the deposition of cholesterol in the aorta of rabbits fed a diet of ground oats and carrots plus 1% cholesterol.

In the present studies, 22 female rabbits, 3 to 5 months old, were used. Each was fed a basal diet of Ralston Purina rabbit chow daily. Ten of these served as controls; 4 received 100 mg of *d,l*-alpha-tocopherol in olive oil (1 ml) intramuscularly 3 times weekly for 12 weeks and 6 one gram of cholesterol added to chow 3 times weekly for the same time. Twelve rabbits received both the tocopherol (in olive or sesame oil) and cholesterol.

The whole blood cholesterol was determined at regular intervals by a modified Bloor method.[3] At the end of the experimental period (12 weeks), the rabbits were sacrificed. The procedure used for killing the animal, removing the aorta and determining its cholesterol content has been described elsewhere.[4] An alcohol-ether extract of the wet liver was also analyzed for cholesterol by Bloor's procedure.[3]

Results and Comment. Table I indicates that the parenteral administration of vitamin E augments appreciably the deposition of cholesterol in the aorta of cholesterol-fed rabbits. These results are at variance with those recently published by Dam[2] who did not ob-

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[1] Dam, H., and Kelman, E. M., *Science*, 1942, **96**, 430.

[2] Dam, H., *J. Nutrition*, 1944, **28**, 289.

[3] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

[4] Bruger, M., and Fitz, F., *Arch. Path.*, 1938, **25**, 637.

TABLE I.
Effect of Vitamin E With and Without Feeding of Cholesterol on Cholesterol Content of the Blood, Aorta and Liver of Rabbits.

		Avg whole blood cholesterol (mg/100 ml)					Avg aorta cholesterol g/100 g dry wt	Avg liver cholesterol g/100 g wet wt
Experiment 3 times a week	No. of rabbits	0	2	4	8	12		
				weeks				
100 mg Vit. E intramuscularly	4	95	103	104	82	89	0.360	0.372
1 g cholesterol added to chow	6	101	123	194	329	325	0.897	0.743
100 mg Vit. E intramuscularly and 1 g cholesterol added to chow	12	103	105	285	392	448	2.165	0.794

serve this effect. The apparent discrepancy may be ascribed to the different routes and dosages employed in the two investigations. Dam fed vitamin E in concentrations of 100 mg of *d,l*-alpha-tocopherol per kg of oats-cholesterol mixture; the amount of this vitamin consumed by the rabbits in the experimental period of 85-97 days is not stated. Assuming a rabbit eats approximately 100 g of food per day, each rabbit in his series ingested about 900 mg of this vitamin in 13 weeks. In the present experiments, vitamin E was injected intramuscularly 3 times weekly for 12 weeks in doses of 100 mg per injection, *i.e.*, a total of 3600 mg of vitamin E per rabbit or about four times the amount of vitamin E probably used by Dam.

Conclusion. In cholesterol-fed rabbits, the intramuscular administration of *d,l*-alpha-tocopherol (vitamin E) in olive oil or sesame oil augments appreciably the deposition of cholesterol in the aorta but increases only slightly the concentration of this lipid in whole blood and liver.

Mr. Samuel Member, B.S., and Miss Shirley Wechsler rendered valuable technical assistance. The vitamin E was generously supplied by Hoffmann-LaRoche, Inc., Nutley, N.J., through the courtesy of Dr. William T. Strauss.

14981

Inhibitory Effect of Glycogen upon Anaphylactic Shock in the Rabbit.

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In another paper from this laboratory[1] it was shown that hydatid fluid produces a sharp reduction in rabbit blood histamine and the leucocyte count. Since under urethane anesthesia, a similar decrease in blood histamine occurred, without leucopenia, the conclusion was drawn that both phenomena are independent. As platelets always disappeared from blood after the injection of hydatid fluid, we have concluded that the fall in blood histamine is more likely to depend on a decrease of platelets, additional evidence in favor of the theory proposed by Minard[2] and Zon, Ceder, and Crigler[3] that the main carriers of histamine in rabbit blood are the platelets. Submitting hydatid fluid to further fractionation, we have been able to verify that a macromolecular, polysaccharide fraction is the con-

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* From the Butantan Institute, São Paulo, Brazil.

[1] Graña, A., and Rocha e Silva, M., in press.

[2] Minard, D., *Am. J. Physiol.*, 1941, **132**, 327.

[3] Zon, L., Ceder, E. T., and Crigler, C. W., *Publ. Health Rep.*, 1939, **54**, 1978.

TABLE I.
Influence of Liver and Ascaris Glycogen and a Polysaccharide Fraction from Hydatid Fluid upon Blood Histamine and Leucocytes in the Rabbit.

Rabbit No.	Anesthesia used	Material injected	Blood histamine (γ/1 cc)		W. B. C. ($\times 10^{-3}$)		Effect upon blood pressure
			Before	After	Before	After	
1	None	Hyd. polys.	1.75	0.25	5.8	1.6	—
2	” ”	EE_0 (5cc)*	0.66	0.57	13.2	5.0	—
3	” ”	” ” ” ”	2.30	0.23	8.6	1.6	—
4	” ”	Asc. glyc.	1.97	0.38	—	—	—
5	” ”	” ” ” ”	1.80	0.23	13.8	4.2	—
6	” ”	L. glyc.	1.50	0.27	11.4	4.8	—
7	” ”	” ” ” ”	1.40	0.42	—	—	—
8	Dial + ether	” ” ” ”	1.75	0.81	29.2	3.6	No shock
9	” ” ” ”	” ” ” ”	1.10	0.62	12.4	6.2	” ” ” ”
10	Chloralose	” ” ” ”	1.50	0.28	6.2	6.6	” ” ” ”
11	” ”	” ” ” ”	1.60	0.23	6.4	10.6	” ” ” ”
12	Urethane	EE_0 (5cc)	2.22	0.30	22.6	25.0	” ” ” ”
13	” ”	L. glyc.	1.00	0.25	7.4	10.4	” ” ” ”
14	” ”	EE_0 (5cc)	1.65	0.40	8.8	8.0	” ” ” ”
15	” ”	Asc. glyc.	2.80	0.82	17.2	22.8	” ” ” ”
16	” ”	Hyd. polys.	2.05	1.50	30.0	29.2	” ” ” ”

* EE_0 means deproteinized and dialysed ascaris extract; Asc. glyc., represents the fraction obtained from EE_0 by precipitation with 50% ethyl alcohol; L. glyc. means a highly purified preparation from dog's liver glycogen; Hyd. polys. means the polysaccharide fraction prepared from hydatid fluid. The solid materials were usually injected in the dose of 100 mg in 5 cc of saline.

stituent responsible for this effect upon rabbit blood histamine and leucocyte-platelet count.

In the meantime, we have been studying the anaphylaxis-like reaction produced in dogs and guinea pigs by an Ascaris extract prepared according to Machebouef and Mandoul.[4] This Ascaris extract produces a shock in guinea pigs and dogs, that is indistinguishable from very severe anaphylactic shock. In the rabbit, this Ascaris material produces a sharp fall in blood histamine and leucopenia, but no shock. Concomitantly, the platelets disappear from blood. Under urethane anesthesia, however, there is rather a leucocytosis or no change in the leucocyte count, and yet the blood histamine is sharply reduced. Chemical fractionation of the Ascaris material showed that a glycogen-like material, which can be precipitated by 50% ethyl alcohol, is the constituent responsible for these effects upon blood histamine and the leucocyte-platelet count. This suggested the possibility that liver glycogen might produce a like effect, which is confirmed in the present paper.

Experimental. The rabbits were anesthetized with 1 g of urethane per kilo of body weight, by the intraperitoneal route. For Dial anesthesia, a dose of 0.50 to 0.65 cc of Dial "liquid" (Ciba) was injected with a complement by ether. For Chloralose anesthesia, a dose of 0.11 g per kilo was injected intravenously in 10 cc of warm saline. Duplicate samples (1 cc) of blood for histamine estimation were taken from the jugular vein in the anesthetized animals or from the ear vein or the heart in the unanesthetized animals, before and after the injection of the materials. The histamine extraction for estimation was made by Code's method[5] and the isolated guinea pig ileum used for the assay. For the anaphylaxis experiments, the animals were anesthetized with urethane or Dial + ether and the carotid blood pressure recorded. Sensitization to horse serum followed Grove's schedule.[6] The *Ascaris lumbricoides* were collected from hogs in the slaughter house and the extracts prepared according to Machebouef and Mandoul.[4] This extract (EE_o) produces an enormous emphysema in guinea pigs in doses up to 2 cc and a profound shock in dogs, when injected intravenously in doses up to 3 to 5 cc. The shock produced in guinea pigs and dogs has been studied in our laboratory and the results will be published elsewhere.[7] The extract EE_o contains a considerable amount of a glycogen-like material (positive iodine test), which can be precipitated by addition of an equal volume of absolute ethyl alcohol. This precipitate can be further purified by successive redissolutions and precipitations with 50% ethyl alcohol. The polysaccharide from hydatid fluid (*Toenia echinococcus*) was prepared by precipitating a 10 times concentrated, deproteinized and dialysed fluid, with 70% ethyl alcohol.

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[4] Machebouef, M., et Mandoul, R., *C. R. Soc. Biol.*, 1939, **130**, 1032.

[5] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

[6] Grove, E., *J. Immunol.*, 1932, **23**, 114.

Results. Effect upon in vivo rabbit blood histamine. The experiments described in Table I were performed on 16 normal rabbits. Seven unanesthetized rabbits were intravenously injected with the indicated material and the blood histamine and leucocytes estimated before and after the injection. Microscopic slides were prepared for an evaluation of the presence of platelets. As a rule, the platelets were drastically reduced after the injection of the polysaccharide materials. Injection of 15 to 30 mg of glycogen per kilo of body weight is enough to remove 80 to 90% of the platelets previously present. The duration of this effect varies from 20 minutes to 1 hour, the platelets returning slowly to normal. Higher doses (100 to 200 mg) produce a more pronounced effect, for longer periods of time. There was considerable reduction in both the histamine content and the leucocyte count in every rabbit injected with polysaccharides extracted from Ascaris or hydatid fluid, as well as in those given 100 to 200 mg of purified glycogen. In another group of animals, 2 were anesthetized with dial + ether, 2 with chloralose and 5 others with urethane. As shown in Table I, in the animals submitted to Dial + ether anesthesia, there was the same drop in both the histamine and the leucocyte count, while in the rabbits submitted to chloralose or urethane anesthesia, there was rather a leucocytosis, although the histamine content fell sharply in all cases. In every case, the platelets fell sharply, almost to zero, after the injection of the polysaccharides. In a few animals, estimation of the platelet volume was made by using the thrombocytocrit, according to Van Allen.[8] In one rabbit submitted to urethane anesthesia, the platelet volume fell from 0.35/100 cc of blood, to 0.08/100 cc of blood, while the leucocytes varied only from 7,200 to 6,000. In another rabbit submitted to ether anesthesia, the platelet volume dropped from 0.60/100 cc of blood to 0.05/100 cc of blood, while the leucocytes varied from 10,600 to 1,200, after the injection of 300 mg of liver glycogen.

These findings are quite in agreement with those previously reported by us[1] with hydatid fluid and lend support to the theory that rabbit blood histamine is mostly carried by platelets. In perfect agreement with our previous experiments also, is the fact that this drop in the histamine content of rabbit blood can co-exist with no change in the carotid blood pressure (no shock), as shown in Table I. This definitely shows that the decrease in blood histamine observed in anaphylactic shock in the rabbit by Rose and Weil,[9] Katz,[10] and Dragstedt *et al.*[11] is not sufficient to explain the fall in carotid blood pressure which follows injection of the antigen into sensitized rabbits.

In vitro experiments with samples of rabbit blood. The addition of liver glycogen or the glycogen derived from Ascaris extracts to samples of rabbit blood do not produce a shift of histamine from cells to plasma, as occurs in anaphylactic,[10,11] trypsin,[12] and peptone shocks. This absence of histamine discharge from cells to plasma was also observed with hydatid fluid and appears to demonstrate that the production of an anaphylactic shock in the rabbit is rather concerned with a liberation of histamine from cells to plasma, as shown by Katz[10] and Dragstedt *et al.*[11]

Effect of liver glycogen upon the course of the anaphylactic shock in the rabbit. This extraordinary property of liver glycogen to

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[8] Van Allen, C. M., *J. Lab. and Clin. Med.*, 1926, **12**, 282.

[9] Rose, B., and Weil, P., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 494.

[10] Katz, G., *Science*, 1940, **91**, 22.

[11] Dragstedt, C. A., Arellano, M. R., and Lawton, A. H., *Science*, 1940, **91**, 617.

[12] Dragstedt, C. A., and Rocha e Silva, M., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 420.

TABLE II.
Influence of Liver Glycogen upon the Course of Anaphylactic Shock in the Rabbit.

Rabbit No.	Anesthesia used	Material injected	Blood histamine (γ/1 cc)		W. B. C. ($\times 10^{-3}$)		Intensity of shock
			Before	After	Before	After	
17	Urethane	Glyc. + serum	1.70	0.64	17.0	4.0	±*
18	”	” ”	1.40	0.50	—	—	0
19	Dial + ether	” ”	1.85	0.33	12.0	2.0	+
20	” ”	” (3 g)	1.40	0.07	14.6	4.0	0
”	Same (7′ after)	Serum (4 cc)	0.07	0.12	4.0	2.6	0
21	Dial + ether	Glyc. (3 g)	2.70	0.50	9.1	4.1	0
”	Same (9′ after)	Serum (4 cc)	0.50	0.27	4.1	1.5	0
22	Urethane	Glyc. (0.5 g)	1.35	1.00	9.3	7.5	0
”	Same (2′ after)	Serum (4 cc)	1.00	0.27	7.5	2.6	++
23	Dial + ether	Glyc. (0.5 g)	2.40	2.00	10.9	8.4	0
”	Same (50″ after)	Serum (5 cc)	2.00	0.63	8.4	1.7	+++
24	Dial + ether	Serum (5 cc)	2.40	1.50	9.8	2.8	++
”	Same (2′ after)	Glyc. (0.5 g)	1.50	0.82	2.8	1.7	+++
25	Dial + ether	Serum + glyc.	1.68	0.20	11.6	3.2	++
26	” ”	” ”	2.15	0.73	14.4	7.2	+++

+++, rapidly fatal shock; ++, long standing fall in carotid blood pressure, recovery in 15 to 30 minutes; +, evanescent fall in carotid blood pressure, return to normal in 5 to 10 minutes; ± or 0, slight or absent shock.

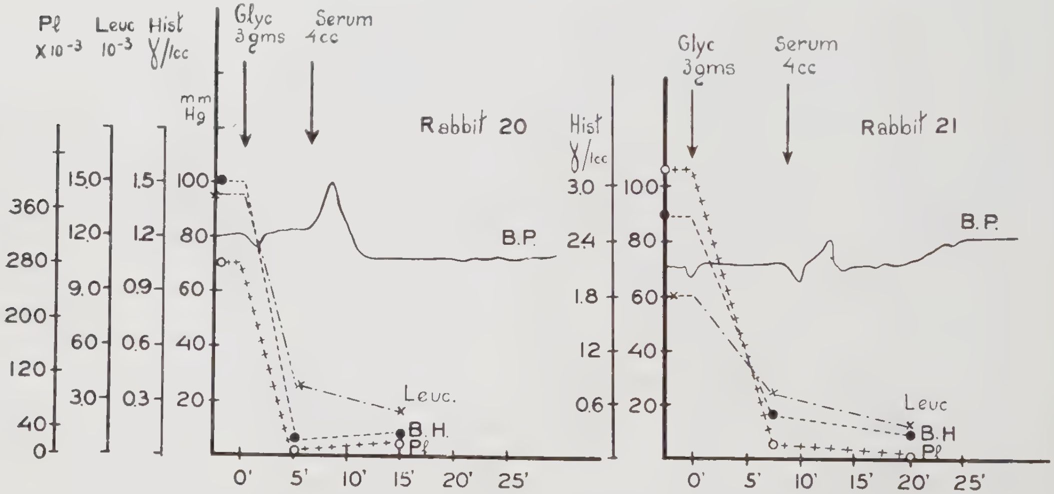

FIG. 1.

Inhibitory effect produced by liver glycogen upon the anaphylactic shock in rabbits 20 and 21. As seen, a previous injection of 3 g of glycogen, 7 to 9 minutes before the injection of serum, produces drastic reductions in blood histamine and the leucocyte-platelet count and prevents the drop in carotid blood pressure, which usually follows the injection of serum in sensitized rabbits.

drastically reduce the histamine from rabbit blood without producing shock or a discharge of histamine from blood elements to plasma, appeared to provide a means to study further the mechanism of anaphylactic shock in the rabbit. This always appeared to be the consequence of a clumping of leucocytes[14] and probably also of platelets in the small vessels and capillaries of the lung, and of a discharge which aggravates the picture, by constricting the pulmonary vessels.[11] Our experiments showing that a considerable reduction in leu-

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cocytes and platelets is not enough to produce shock seem to warrant the idea that the liberation of histamine constitutes a very important factor in localizing to the lung capillaries the emboli produced by the clumping of blood elements.

In 6 rabbits we have studied the effects of a previous injection of glycogen upon the course of anaphylactic shock and in 5 others, glycogen was given after the injection of the antigen. It became apparent (Table II and Fig. 1) that if a proper amount of glycogen is injected (1 to 3 g) and enough time is allowed to elapse (5 to 10 minutes) before injection of the serum, the blood histamine, the leucocytes, and the platelets are drastically reduced and shock will not occur after the injection of serum. This is clearly shown in rabbits 20 and 21. When an insufficient amount of glycogen is injected 1 to 2 minutes before the serum, the shock which occurs is either milder or unchanged. Given after the serum, glycogen rather aggravated the condition or had no effect at all.

Interesting enough is the fact that the leucopenia in anaphylactic shock probably derives from a different mechanism than that produced by glycogen, since under urethane anesthesia, glycogen produces a leucocytosis or no change in the leucocyte count, while anaphylactic shock produces a drop in leucocytes even under these anesthetics. Since histamine is bound to platelets, it seems logical to conclude that the reduction in blood histamine during anaphylactic shock in the rabbit depends upon the removal of the platelets from circulating blood, this being an inevitable occurrence during this kind of shock.[15]

Summary. Polysaccharides prepared from *Ascaris lumbricoides* and hydatid fluid, as well as liver glycogen, produce a sharp reduction in blood histamine in the unanesthetized rabbit or in those under Dial + ether anesthesia, urethane and chloralose anesthesia. Concomitantly, platelets almost disappear from circulating blood while the leucocytes are reduced only in the unanesthetized rabbit or in those under Dial + ether anesthesia. Under chloralose or urethane anesthesia, there was rather a leucocytosis after the injection of glycogen. Thus the conclusion was drawn that histamine is bound to platelets in rabbit blood. Glycogen has a definite inhibitory effect upon the course of anaphylactic shock, explained by the fact that glycogen *disperses* the blood elements, without a preferential allocation of those in the shock organ, while anaphylactic shock in the rabbit occurs as a consequence of the clumping of leucocytes and platelets in the lung capillaries and small vessels, which become a filter for agglutinated blood elements.

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[15] Kopeloff, N., and Kopeloff, L. M., *J. Immunol.*, 1941, **40**, 471.

We are indebted to Drs. W. O. Cruz and E. Martins, of the Oswaldo Cruz Institute, in Rio de Janeiro, who kindly helped us in the experiments with the Van Allen's thrombocytocrit, in the hematological laboratory at the O. Cruz Institute.

14982 P

Alloxan in Pregnant Rats.*

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Diabetes mellitus can be produced in rats by the injection of alloxan.[1] Although there is slight damage to other tissues of the body, the principal action of alloxan is the production of a selective necrosis of the beta cells of the islets of Langerhans. The diabetic state produced can be ameliorated by adrenalectomy[2] and by insulin therapy.[3]

In an attempt to produce offspring with

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* This study was aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **245**, 384.

[2] Janes, R. G., and Friedgood, C. E., *Endocrinology*, 1945, **36**, 62.

[3] Kaplan, N. O., Franks, M., and Friedgood, C. E., *Science*, 1945, in press.

diabetes mellitus, we undertook studies to determine whether alloxan passed through the placenta in quantities sufficient to produce the disease. Although some evidence seems to indicate that islet cells do not function before birth[4] we investigated what effect maternal hyperglycemia might have on the islet cells of the fetus.

Methods. (A) *Experiments to Determine Placental Transmission.* Sixteen rats, which were 18 to 20 days pregnant, were anesthetized (14 with pentobarbital intraperitoneally; 2 with vinethene inhalation). The abdomen was then opened, and the uterus incised longitudinally along the anti-mesometrial surface. Amniotic fluid was aspirated from several of the vesicles and these fetuses were then decapitated to obtain blood samples. Maternal blood was also drawn. These samples served as controls. A 5% solution of alloxan (200 mg/kg) was then injected into the inferior vena cava of the mother. Amniotic fluid was aspirated from the remaining vesicles one minute after the start of the alloxan injection, and the fetuses were removed and decapitated. The blood was collected on a watch glass, moistened with a 1% solution of heparin. Simultaneously a maternal blood sample was taken from the renal vein. Each of these samples was added to a strong tungstic acid solution. The filtrate separated from the tungstic acid precipitate was tested for the presence of alloxan by the qualitative method described by Leech and Bailey.[5]

(B) *Experiments on Maternal and Fetal Hyperglycemia.* Blood sugar studies were done on the following:

(1) Five normal 20-day-pregnant rats and their fetuses.

(2) Three normal mothers 1-2 day post-partum and their offspring.

(3) Three 20-day-pregnant diabetic mothers and their fetuses.

(4) One diabetic mother 1 day post-partum and her offspring.

The mothers made diabetic were given alloxan intraperitoneally (200 mg/kg in 5% solution) on the fourteenth day of gestation, and by the seventeenth day they showed polyphagia, polydypsia, marked polyuria (as high as 150 cc per 24 hours), a heavy 4+ glycosuria, and some had ketonuria.

Rats used in both experiments were from the Long-Evans strain.

Results. The control blood samples from both mother and fetus showed negative tests for alloxan. A faint blue reaction was detected in the control amniotic fluid; however, the substance giving this reaction was not determined. Following the injection of alloxan, a strong positive test for alloxan was obtained in the fetal as well as in the maternal blood. The amniotic fluid specimens drawn after the injection of alloxan gave a slightly more intense positive reaction than the control sample, but much less intense than the positive fetal blood specimens.

Blood sugar samples of the normal pregnant and postpartum mothers averaged 128 mg% (range 112-136 mg%). The blood sugar of the fetuses and of litters averaged 120 mg% (range 108-129 mg%).

Blood sugar values of the pregnant diabetic rats averaged 366 mg% (range 344-418 mg%). Their fetuses showed a slightly lower blood sugar value—average 327 mg% (range 292-373 mg%).

The diabetic maternal blood sugar one-day postpartum was 352 mg% while that of her offspring was 124 mg%.

When alloxan was injected into pregnant rats 6 hours to 4 days before parturition the mothers showed typical signs of diabetes mellitus whereas the litters were not diabetic. If the litters were placed with normal foster mothers, they developed normally, with no signs of diabetes mellitus, and the pancreatic islets showed no definite pathological change.

Summary. 1. In pregnant rats intravenously injected alloxan passes through the placenta into the fetal circulation within one to two minutes. 2. It does not, however, produce permanent diabetes in the offspring. 3. The blood sugar values of the fetus parallels at a lower level that of the diabetic mother until parturition.

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14983

Effect of the Host in Action of Sulfonamides on Elementary-Body Agents of Murine and Feline Pneumonitis.*

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The effect of sulfanilamide and its derivatives on experimental infections with 4 agents of the lymphogranuloma-psittacosis group has been studied. Infections in mice with 2 of these agents, mouse pneumonitis and lymphogranuloma venereum, are inhibited with sulfonamides,[1,2] while infections with the agents of feline pneumonitis[3] and meningopneumonitis[4] are not.[2,5] Growth of the latter virus and psittacosis virus in chick embryos or mice is retarded by penicillin.[6,7] Clinical observations suggest that the sulfonamides have no effect in human infections with psittacosis and closely related agents.[8]

A close biological similarity of the agents from mice and cats and the formation of a complement-fixing antigen which cross-reacts with other members of the lymphogranuloma-psittacosis group have been noted.[9,10] Both agents produce pneumonia of almost identical character in mice inoculated intranasally, but neither agent will produce lethal infection in these animals by any other route except when large doses of yolk sac material are inoculated.[5] These properties distinguish the two agents from the other members of this group which produce fatal infections in mice inoculated intracerebrally.

Three similar strains forming elementary bodies, 2 from mice and one from hamsters,[11] were, like the original strains of mouse pneumonitis, inhibited by sulfonamides.[12] Since the biologically similar agent from cats was apparently exceptional in being drug resistant, it seemed possible that an investigation of the differences between resistant and susceptible strains might give some information about mechanisms of chemotherapy of certain virus diseases. One line of approach was to compare the cat and mouse viruses in several different hosts.

Materials and Methods. Sulfamerazine† and other relatively insoluble drugs were suspended in 10% gum acacia and injected intraperitoneally. Mice, rats, cotton rats (*Sigmodon hispidus eremicus*), hamsters (*Cricetus auratus*), and chick embryos were used in the experiments. Generally the animals were weighed and the dosage adjusted in relation to body weight. The strain of mouse pneumonitis virus was originally isolated by Dr. Clara Nigg who kindly sent it to us. The feline pneumonitis virus was obtained from Dr. James A. Baker of the Rockefeller Institute at Princeton, New Jersey. Both strains produced characteristic focal pulmonary lesions[3,11] when given intranasally in high dilutions, and the character of these lesions was the same in

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* The studies and observations on which this paper is based were supported by the International Health Division of the Rockefeller Foundation, in cooperation with the California State Department of Public Health.

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† 2-sulfanilyl aminomethylpyrimidine. Manufactured by Parke, Davis & Co.

TABLE I.
Effect of Sulfamerazine on Infections with the Agent of Mouse Pneumonitis.

		Daily drug dosage			Results with test animals		Results with controls	
Animal	Dilution of virus	mg/g	No. days	Day killed	Lesions*	Avg lung wt (g)	Lesions*	Avg lung wt (g)
Hamster	10^{-1}	0.3	3	4	3 3 2 2	2.30	5 5 4 3	2.6
,,	10^{-3}	0.3	7	7	1 0 0 0 0	0.75	2 2 1	1.0
,,	10^{-2}	0.1	3	4	1 1 0 0	0.90	3 3 2 2	2.05
,,	10^{-3}	0.1	3	7	0 0 0	—	3 3 2	—
,,	10^{-4}	0.1	3	7	1 0 0	—	1 1 1	—
,,	10^{-2}	0.025	3	4	3 2 2 0	1.40	3 3 2 2	2.05
Cotton Rat	10^{-3}	0.3	6	6	1 0 0 0 0 0	0.90	3 2 2 1	1.70
Rat	10^{-2}	0.6	7	8	1 1 0 0	1.50	5 5 2 1	2.60
Mouse	10^{-1}	0.1	4	4	0 0 0 0 0 0	—	5 5 5 5 5 5	—
,,	10^{-2}	0.1	4	4	0 0 0 0 0 0	0.166	3 3 3 3 3 3	0.300
,,	10^{-3}	0.01	3	7	0 0 0 0 0 0	—	2 2 2 2 2 2	—
,,	10^{-3}	0.005	3	7	1 0 0 0 0 0	—	same	—
,,	10^{-3}	0.0025	3	7	1 1 f 0 0 0	—	,,	—

* Pulmonary lesions are shown by numeral for each animal and were graded as follows: 5 = animal died with pulmonary consolidation; 4, 3, or 2 = 4/4, 3/4, or 2/4 of lung consolidated; 1 = ⅛ to ¼ of lung consolidated; f = 5 to 20 minute discrete foci; 0 = no lesion.

the various species of animals used in the experiments. Elementary bodies were demonstrable in impression smears stained by the method of Macchiavello. The viruses were inoculated intranasally in dilutions of mouse lung containing 10 to 1,000 times the minimal amount required to produce pulmonary lesions within a period of 7 days. In all experiments the virus was inoculated 2 to 4 hours after the first dose of drug had been given intraperitoneally and drug was administered daily thereafter. Pulmonary lesions were graded by gross examination and by weighing the lungs. The effect of variations in body weight was minimized by selecting the animals so that average weights of the test group and the control group were approximately equal.

Chick embryos 6 to 7 days old were inoculated into the yolk sac with egg-adapted agents of feline and murine pneumonitis, and a single dose of drug was given by the same route either before or after the virus. All embryos were cultured in glucose broth for bacterial contamination either at death or

TABLE II.
Effect of Sulfamerazine on Infections with the Agent of Feline Pneumonitis.

		Daily drug dosage			Results test animals		Results controls	
Animal	Dilution of virus	mg/g	No. days	Day killed	Lesions	Avg lung wt (g)	Lesions	Avg lung wt (g)
Hamster	10^{-3}	1.0	4	6	1 f f 0 0	—	3 3 2 2 2 2	—
,,	,,	0.6	6	6	f 0 0 0 0	0.95	2 2 1 1 1	1.19
,,	10^{-4}	0.6	6	7	f f 0 0 0	0.76	f f f f 0 0	0.78
,,	10^{-3}	0.3	5	5	3 1 1 1 1 f	1.30	3 3 3 3 2	1.60
,,	10^{-4}	0.3	5	5	1 1 1 f f 0	0.85	1 1 1 1 1 1	0.97
,,	10^{-3}	0.3	6	7	1 1 1 1	0.85	3 2 2 1	1.10
Cotton Rat	10^{-4}	0.6	6	7	1 1 1 1 f f	1.33	3 3 2 2 2 1	1.59
,, ,,	10^{-4}	0.3	5	5	2 2 1 1 1 1	1.17	3 2 2 2 2 2	1.34
,, ,,	10^{-4}	0.4	7	7	5 2 2 2 1 1	1.36	5 4 3 3 3 2	1.53
,, ,,	10^{-3}	0.3	6	6	5 3 3 2	1.75	5 5 3 3	2.20
Rat	10^{-2}	0.6	7	8	1 1 1 f f f	1.50	5 5 3 2 2 1	3.10
,,	10^{-2}	0.6	8	9	f f 0 0	1.10	1 f f 0	1.20
Mouse	10^{-3}	1.2	6	8	5 3 3 3 3 2	—	5 3 3 3 2 2	—
,,	10^{-4}	1.2	6	8	2 2 1 1 1	—	2 2 2 2 1 1	—
,,	10^{-3}	0.6	6	6	3 3 3 3 2 2	0.38	3 3 3 2 2 1	0.30
,,	10^{-4}	0.6	6	6	3 3 1 1 1 1	0.23	3 3 2 1 1 1	0.25
,,	10^{-5}	0.6	6	7	1 1 1 1 1 1	0.25	1 1 1 1 1 f	0.25

Lesions graded as in Table I.

TABLE III.
Blood Concentrations of Sulfamerazine After Single Daily Intraperitoneal Injections.

Animal	No. tested	Average sulfamerazine blood-levels mg/100 cc 1st day dose = 0.9 mg/g Time after injection 4 hr	 24 hr	 2nd day dose = 0.6 mg/g Time after injection 4 hr	 24 hr
Cotton rat	4	20.4	4.8	18.0	5.3
Hamster	4	17.8	6.3	15.8	3.5
Rat	7	39.8	21.6	43.2	21.2
Mouse	12	34.5	11.1	42.2	13.9

after the termination of the experiment.

Effect of Sulfonamides on the Mouse Pneumonitis Virus in Animals. Using the method of single daily intraperitoneal dosage over a period of 4 days, the minimal inhibiting dose of sulfanilamide and 4 of its derivatives against a constant amount (dilution 10^{-3}) of the mouse pneumonitis virus was determined in mice. Under these conditions the minimal inhibiting dose of sulfanilamide was found to be 1.0 mg per gram of body weight; that of sulfapyridine 0.30 mg/g, sulfathiazole 0.15 mg/g, sulfadiazine 0.005 mg/g, and sulfamerazine 0.0025 mg/g per day. Measurements of the blood levels at intervals after giving the drugs indicated that the rather marked differences in the effective amounts could be attributed, at least in part, to the rate of absorption and excretion of these compounds. After intraperitoneal injection of sulfamerazine appreciable blood levels remained after 24 hours, while drugs such as sulfanilamide and sulfathiazole were excreted more rapidly. Sulfamerazine was, therefore, chosen for use in the experiments to be described.

The results presented in Table I show that sulfamerazine inhibits infections with the mouse pneumonitis agent in mice, rats, hamsters and cotton rats. Small doses of drug (0.1 to 0.025 mg/g) were less effective in preventing the appearance of lung lesions in hamsters than in mice when the animals were inoculated intranasally with dilutions of 10^{-2}. Similarly, with virus dilutions of 10^{-1} a drug dosage of 0.1 mg/g gave complete inhibition in mice, while in hamsters 0.3 mg/g failed to produce significant reduction in the lesions. As the virus titered slightly higher in mice than in hamsters the greater effectiveness of the drug in mice probably cannot be attributed to a lower susceptibility of these animals.

Experiments with the Agent of Feline Pneumonitis in Animals. Tests similar to those with the agent of mouse pneumonitis were done with the virus of feline pneumonitis, with the results shown in Table II. In hamsters, sulfamerazine in amounts of 0.6 to 1.0 mg per gram of body weight daily produced a moderate inhibitory effect and 0.3 mg/g had a slight effect on the pulmonary lesions resulting from inoculation of virus at a dilution of 10^{-3} or 10^{-4}. This drug dosage was about 6 to 10 times as great as the amount required to produce a comparable effect on the agent of mouse pneumonitis in hamsters. In cotton rats a slight effect was noted, particularly with drug in amounts of 0.6 mg/g and a virus dilution of 10^{-4}. In 2 experiments with white rats an inhibitory effect was found, but the results were unsatisfactory because the control animals receiving a dilution of 10^{-2} in one experiment did not develop definite lung lesions. No inhibitory effect was found in mice even with doses of sulfamerazine of 1.2 mg/g, an amount between 200 and 500 times as great as that producing inhibition of the mouse pneumonitis virus in these animals. It should be noted that the agent of feline pneumonitis was not significantly more virulent for mice than for cotton rats or hamsters, as judged by titration.

Blood Levels with Sulfamerazine. The possibility that the species differences in the effect of sulfamerazine on the feline pneumonitis virus might be connected with the blood levels was considered. The results of determining sulfamerazine concentrations in the blood at 4 and 24 hours after a single daily intraperitoneal injection, as shown in Table III, indicated no relationship between the blood levels

TABLE IV
Effect of Sulfamerazine on Yolk-Sac Infection of Chick Embryos with the Agents of Murine and Feline Pneumonitis.

Virus	Dilution	Drug dosage, mg	Time	Day of death of chick embryos Drug treated	Day of death of chick embryos Controls
Mouse Pn.	10^{-2}	20	3 hr after	6 S S S S S S*	4 4 4 4 5 5 5 6
Mouse Pn.	10^{-2}	4	3 hr after	8 8 9 S S S S S*	4 4 6 6 6 6 6 6
Cat Pn.	10^{-7}	20	3 hr after	6 6 6 6 6 6 6 10	6 6 6 6 6 6 6 6
Cat Pn.	10^{-7}	20	2 hr before	5 6 6 6 6 6 6 6	4 6 6 6 6 6 6 6

S—Survived 10 to 12 days.
*—Virus detected in surviving chick embryos by subinoculation of mice.

and the effect on the cat virus. In mice no inhibitory effect was observed, although the blood concentration was on the average almost twice as great as in hamsters or cotton rats. On the other hand, the differences in blood levels between mice and hamsters might account for the observation that relatively more drug was required in hamsters to inhibit the mouse pneumonitis virus.

Experiments in Chick Embryos. At a period of 10 days after inoculation of the embryos with a single dose of 20 mg of sulfamerazine into the yolk sac the drug was found in concentrations of 7 mg% in the amniotic fluid, 26 mg% in the allantoic fluid, and 16 mg% in the yolk and blood. This observation indicated that only one dose was required. Doses of 20 mg produced almost complete protection and doses of 4 mg partial protection against death of the embryos from a dilution of 10^{-2} (over 1,000 M.L.D.) of the mouse pneumonitis virus when given 3 hours after inoculation of the virus (Table IV). No inhibition of the infection with the virus of feline pneumonitis at a dilution of 10^{-7} (1 M.L.D.) was obtained with sulfamerazine given before or after inoculation of the virus. This drug also failed to inactivate the virus at a dilution of 10^{-3} in egg yolk when mixtures were allowed to stand for 4 hours *in vitro* at 37°C before inoculation of the chick embryos.

Discussion. The difference in sensitivity to sulfamerazine between the pneumonitis viruses from cats and mice appears to be quantitative in nature. In hamsters the inhibiting dose of drug for the cat agent was about 10 times that for the mouse virus. In mice the comparable ratio was found to be over 200 to 1 as determined by two factors: (1) maximum tolerated doses of drug failed to inhibit the cat virus, and (2) a smaller dose than required in hamsters would protect mice against the mouse virus. The differences between the two viruses are analogous to the phenomenon of drug resistance or sensitivity often observed with strains of bacteria in one species and might be due to similar factors. The results also suggest that unknown physiological mechanisms in the host may influence the effect of the sulfonamides (1) on the virus within the cells or (2) on extracellular virus either during the stage of initial infection or during spread of the virus particles from one cell to another. On the basis of the available data this effect does not seem to be related to the concentration of sulfamerazine in the blood or to the minimal infecting dose of the agent.

Summary. The effect of sulfamerazine on pulmonary infections in mice, hamsters, rats, and cotton rats, and on yolk sac infections in chick embryos with the agents of murine and feline pneumonitis was studied. The drug inhibited the action of the mouse virus in all the species tested and was effective in very small doses, 0.0025 mg per gram of body weight in mice and somewhat larger doses, 0.1 mg/g, in hamsters. With the agent of feline pneumonitis, large doses of sulfamerazine, near the toxic limits, produced moderate inhibition of the lung lesions in hamsters and white rats, slight inhibition in cotton rats, and no effect in mice or chick embryos.

14984 P

Effect of Epinephrine on Adrenal Cholesterol and Ascorbic Acid.*

C. N. H. LONG AND E. G. FRY.

From the Department of Physiological Chemistry, Yale University.

It has previously been shown that the injection of pure adrenotrophic hormone into rats is followed by a rapid fall in adrenal ascorbic acid, a slower fall in adrenal cholesterol, and an increase in liver glycogen.[1,2,3] It has been found (unpublished experiments) that exposure of rats to such conditions as cold, scalds, or hemorrhage is also followed by a depletion of adrenal cholesterol and ascorbic acid. However, since the above stresses are without any effect on the adrenal cholesterol and ascorbic acid of hypophysectomized rats, it is evident that a release of adrenotrophic hormone from the anterior pituitary is a preliminary and necessary step in the adrenal cortical response to an increased need for cortical hormone.

The factors, humoral or nervous, that are associated with stress and which lead to an increased secretion of adrenotrophic hormone are not known, but it would appear that one common denominator is an increased degree of activity of the sympathetic nervous system, with a consequent release of epinephrine. We have, therefore, investigated the effect of epinephrine on the cholesterol and ascorbic acid levels of normal and hypophysectomized rats.

Methods. Male rats about 200 g in weight of either Sprague-Dawley or Yale strain were used for this investigation. Non-fasted animals were hypophysectomized by the parapharyngeal approach under ether anesthesia. They were used on the third postoperative day after a fast of 18 hours, while food was withheld from normal rats 24 hours before the experiment.

A 4-hour course of injections was given subcutaneously to both groups. Every hour each animal received 0.02 mg/100 g of epinephrine in physiological saline. One hour following the last injection the rats were quickly anesthetized with nembutal. The adrenals were removed and weighed on a torsion balance. The left adrenal was analyzed for cholesterol and the right one for ascorbic acid. The effect of a single subcutaneous dose of epinephrine of the same concentration was also studied 2 hours after injection.

In another series of experiments the left femoral vein was exposed under nembutal anesthesia, and epinephrine in saline (containing 20 mg% glutathione) was delivered into the circulation at a constant rate of 0.02 mg/100 g/hr for a period of 2 hours, at which time the adrenals were analyzed.

Cholesterol was determined by the Sperry and Schoenhemier method.[4] Ascorbic acid was first determined by Tillman's method and later by the Roe and Kuether procedure.[5]

Results. Table I shows that epinephrine administered subcutaneously causes an unmistakable fall in adrenal cholesterol and ascorbic acid and that this effect is abolished by hypophysectomy. Similar results were obtained with epinephrine administered intravenously. Consequently, the absence of any effect of epinephrine on hypophysectomized rats is not due to failure of absorption from the subcutaneous tissues.

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TABLE I.

	No. animals	Duration of experiments hr	Adrenal Cholesterol, g %	Adrenal Ascorbic acid, mg %
Normal.				
Controls	23	—	4.03 ± 0.10	370 ± 14
Epinephrine. 1 × 0.02 mg/100 g subcut.	6	2.0	2.88 ± 0.28	210 ± 24
Epinephrine. 4 × 0.02 mg/100 g subcut.	10	4.0	2.26 ± 0.22	216 ± 9
Epinephrine. 0.02 mg/100 g/hr intraven.	6	2.0	2.31 ± 0.18	104 ± 17
Hypophysectomized.				
Controls	4	—	5.74 ± 0.43	398 ± 38
Epinephrine. 4 × 0.02 mg/100 g subcut.	9	4.0	5.34 ± 0.47	411 ± 13
Epinephrine. 0.02 mg/100 g/hr intraven.	2	2.0	6.04	357

At the moment it is not possible to decide whether this effect of epinephrine is due to a direct stimulation of the cells of the anterior pituitary that secrete adrenotrophic hormone or whether it is due to changes in the composition of the blood acting as the exciting agent. Further experiments to clarify this question are in progress.

14985

The Incidence of Toxoplasmic Infections in the St. Louis Area.*

William P. Callahan, Jr. (Introduced by Robert A. Moore.)

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Since 1940 8 fatal cases of toxoplasmosis have been observed in the St. Louis area.[1,2,3] Two of the patients were adults, in whom the infection occurred as an acute febrile disease manifested by a skin rash and closely simulating the typhus spotted fever group of diseases. A history of tick bite was obtained from both patients.[1] The other instances of this type of infection have been observed in infants in whom the onset of clinical signs and symptoms was apparent at birth or appeared shortly thereafter.[2,3] Widespread involvement of the central nervous system gave rise to the principal clinical manifestations of generalized convulsions, muscular paralysis and internal hydrocephalus. Cerebral calcification and widespread encephalomyelitis with the formation of many granulomatous lesions were the prominent pathologic changes. Toxoplasma were present in small aggregates or pseudocysts and as free organisms within the central nervous system.

The onset of the disease at birth or shortly thereafter, as well as the advanced nature of the lesions in the central nervous system, led Paige, Cowen, and Wolf[4] to advance the concept of the intrauterine inception of the disease. In the cases reported by these observers, as well as many other instances of the infantile type of toxoplasmic encephalomyelitis, the infection in the mother produced no clinical manifestations of disease although neutralizing antibodies were demonstrated in the peripheral blood.[5,6,7] This would indicate

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that inapparent or subclinical infections may be present and unrecognized unless pregnancy occurs and the infection is transferred to the fetus where it produces irreparable damage.

Since a relatively large number of instances of the infantile type of toxoplasmic infection have been observed in the St. Louis area it may be postulated that inapparent infections in adults may be common. In an attempt to determine the incidence of toxoplasmic infections in this vicinity, neutralization tests were performed on the blood serum of 100 apparently normal individuals between the ages of 18 and 28 years. Because of the possible intrauterine inception of the disease it was thought that a survey including individuals in the child-bearing age from St. Louis should be made, using as a control group persons of the same age from a different locality. Blood was obtained from 26 unmarried girls between the ages of 17 and 24 years who lived outside of the state of Missouri; from an equal number of unmarried girls in the same age group who had been residents of St. Louis since birth; and from 25 married women in the same age group who had one or more normal children. Blood was also obtained from 23 men between the ages of 20 and 28 years.

Blood. Blood was drawn from the antecubital vein under sterile conditions. In most instances, because of the difficulty in obtaining out-of-state residents, a large number of blood samples were drawn at the same time. The blood was placed in sterile Kolmer tubes and allowed to clot at room temperature for 20 minutes. It was then centrifuged for 10 minutes and the serum withdrawn by means of a sterile pipette and placed in soft glass vials. These vials were sealed, labeled and rapidly frozen. They were stored in an insulated box containing solid carbon dioxide. The serum obtained from each individual was then placed in at least 2 vials to allow repetition of the test whenever necessary. Whenever possible, serum was used the day it was obtained and was stored in a refrigerator at 4°C. Under no circumstances was the serum allowed to stand in a refrigerator, without freezing, for more than 24 hours.

Animals. Well nourished, healthy rabbits weighing 3-5 kg were used. Animals with clear skin were selected and preferably white New Zealands were used because of the uniform color of the skin and the ease with which the subsequent reactions could be read. No naturally immune rabbits were encountered. All animals, with the exception of 2, were obtained from one dealer. The backs of the animals were clipped with OO Oster clippers and then carefully shaved with a straight razor. The shaved area was then carefully marked with India ink into 20 squares.

Organism. Through the courtesy of Dr. Abner Wolf the B.D. strain of Toxoplasma, isolated from a fatal human case, was obtained. The organism was maintained by intracerebral inoculation of mice with a 10% suspension of infected mouse brain in veal infusion broth. The organism was passed serially for one month, before it was used for neutralization tests, to determine the relative pathogenicity of this strain. It was found that intracerebral inoculation of .04 cc of a 10% mouse brain suspension produced convulsive seizures on the fourth day and was uniformly fatal on the fifth day. The brains of the mice succumbing on the fifth day after intracerebral inoculation were cultured and found to be bacteriologically sterile. Touch preparations stained by the Wright and Giemsa methods were examined to make certain of the presence of large numbers of Toxoplasma.

Neutralization Test. The method used is similar to that described by Sabin[6] except for several minor alterations. Two whole mouse brains, removed under sterile precautions from infected animals on the fifth day, were ground in a mortar without an abrasive and enough veal infusion broth was added to make a 10% suspension. This was allowed to sediment spontaneously for 15 to 20 minutes in a refrigerator at approximately 6°C. Cultures were made on rabbit's blood agar to determine the presence of any contaminating bacteria. The supernatant fluid was regarded as a 1:10 dilution. From this 1:50, 1:100, 1:500, and 1:2500 dilutions were made using veal infusion broth as the diluent. An equal amount of the undiluted serum to be tested was added to each dilution of the organism making mixtures of 1:20, 1:100, 1:200, 1:1000 and 1:5000. After thorough mixing they were allowed to stand at room temperature for 30 minutes. Two hundredths cubic centimeters of each mixture was then injected intracutaneously into the skin of the rabbit within the previously marked squares.

Interpretation of Neutralization Test. Human sera produced erythema in the rabbit's skin within 24 hours, but the zone of redness disappeared by the third or fourth day. The lesions produced by Toxoplasma appeared by the end of the fifth day and consisted of maculopapular eruptions surrounded by a zone of erythema. By the eighth day the lesions resulting from the larger concentrations of organisms underwent central necrosis and the results were determined on the ninth day. Each lesion was accurately measured, drawn on transparent paper and described. Careful records were kept of the time of disappearance of the erythema, the appearance of the toxoplasmic lesions and the interval to the production of necrosis, to evaluate any individual variations in the type of reaction on different rabbits. By the fourteenth day the lesions produced by the more dilute mixtures began to blanch and the necrotic lesions became encrusted. The animals usually died by the twenty-first day. Any reactions which could not be interpreted as conclusive were repeated on other animals.

Results. The serum of 2 individuals prevented the production of necrotic lesions in the skin of rabbits. These 2 persons were residents of the St. Louis area and had lived in this vicinity all their lives. Careful physical examinations were performed and were entirely negative. Examination of the eye grounds showed no pathologic lesions. A complete medical history was taken and no history of present or past illness was obtained that might be interpreted as a toxoplasmic infection. Both individuals were unmarried girls of 18 and 21 years respectively. No positive tests were obtained from the group of unmarried non-residents, the married women, or from any of the men tested in this series.

Conclusions. The sera of 2.7% of the individuals in the St. Louis area that were tested showed the presence of a definite inhibitory factor on the necrotizing action of Toxoplasma in the skin of rabbits.

14986 P

Human Thyrotoxicosis: Response to Para-Aminobenzoic Acid.

Louis Berman. (Introduced by S. Ansbacher.)

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The use of thiourea and especially of thiouracil in the treatment of hyperthyroidism was first suggested by Astwood.[1] Apparently, these compounds prevent thyroid hormone synthesis and numerous papers[2] have appeared showing that they are effective in mild or moderately severe cases of thyrotoxicosis and in the preoperative preparation of patients for thyroidectomy.[3] The disagreeable taste of thiourea makes thiouracil the preferred therapeutic agent, although the latter has been demonstrated clinically to give untoward reactions, including agranulocytosis,[1] myxedema,[4] skin eruptions, fever, and jaundice.[5,6] Indeed, according to a most recent report,[7] "Thiouracil is an effective drug for the treatment of thyrotoxicosis. It is also an unpredictable toxic drug which may produce serious and uncontrollable effects, especially on the bone marrow and liver."

In view of the ever increasing clinical appli-

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TABLE I.
PABA Treated Hyperthyroid Cases.

Case No.	Age in years, sex	PABA treatment	Observation, Mo.	Weight (kg)	Pulse per min.	Blood pressure, mm	Basal metabolic rate	Serum cholesterol, mg/100 ml	Symptom-free after withdrawal of drug, Mo.
1	34 ♀	½ g every other day 1 week, then 1½ g 6 times weekly 6 mo.	0	50	110-140	150/90	+42	116	15
			1	55	95-110	140/90	+28	125	
			3	60	90-100	135/85	+12	142	
			6	65	80	125/80	— 5	180	
2	48 ♂	1 g 6 times weekly 2 mo., then 1½ g 6 times weekly 4 mo.	0	57	120	130/55	+32	120	11
			2	57	105	130/65	+20	186	
			4	65	85	125/70	+16	202	
			6	69	75	125/80	+ 8	252	
3	18 ♀	1½ g 6 times weekly 9 mo.	0	45	120-150	110/45	+48	95	6
			2	48	100-110	135/70	+25	132	
			4	53	95-105	130/75	+22	138	
			7	57	85-90	130/80	+15	150	
			9	63	80	120/80	+10	162	
4	28 ♀	1 g 6 times weekly 2 mo., then 1½ g 6 times weekly 3 mo.	0	49	120	140/65	+30	159	6
			2	54	100	140/70	+22	170	
			5	64	85	120/75	+ 8	225	
5	36 ♀	1 g 6 times weekly 3 mo., then 1½ g 6 times weekly 5 mo.	0	48	95-145	165/85	+45	170	4
			3	53	95-110	150/85	+32	195	
			5	60	95	145/85	+20	208	
			8	65	80	135/80	+10	252	
6	42 ♀	1 g 6 times weekly 2 mo., then 1½ g 6 times weekly 5 mo.	0	55	110-140	115/55	+36	158	6
			2	61	95-105	110/60	+22	175	
			4	69	85-95	115/70	+20	182	
			7	70	70	125/70	— 5	205	

cation of chemical substances in the treatment of toxic goiter, the results obtained with para-aminobenzoic acid (PABA) are deemed of value to the investigators and clinicians in the field. Although PABA had been investigated experimentally,[8] there have been no reports as to its use in human thyrotoxicosis. The present communication sketches 3 cases with some detail and gives numerical data obtained with 6 patients in tabular form.

My interest in the use of PABA for this purpose was aroused during the first half of 1943 when I began employing its sodium salt parenterally in large doses in the treatment of a case of vitiligo.[9] The patient was a 34-year-old woman (See Table I, Case 1-F.H.) who had lost about 10 kg in the course of a year. Because of a few small patches of vitiligo on her hands, she was given injections of ½ g of the sodium salt of PABA every other day for about one week and then 1½ g 6 times weekly. Her restlessness and nervousness subsided significantly after about 2 weeks of the daily therapy, then her sleeplessness improved with a concurrent gradual gain in weight. Her basal metabolic rate (BMR) had dropped from +42 to +28 by the end of the first month. It was +12 after 3 months and she had gained 10 kg in weight. At the end of ½ year of the treatment, the patient was discharged, her BMR was recorded as —5, her total weight gain was 15 kg and her serum cholesterol had risen from an initial 116 to 180 mg/100 ml. The patient was seen several times during the 15 months following withdrawal of PABA and no signs of recurrence of toxic goiter were observed.

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[8] Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

[9] Sieve, B. F., *Virg. Med. Mo.*, 1945, **72**, 6:

Case 3 (M.R.) was first seen in October, 1943, complaining of swelling of the neck, extreme nervousness, heart palpitation, a peculiar feeling of constant, unmotivated anxiety and crying spells for a period of about a year. She showed a markedly enlarged thyroid with characteristic exophthalmos. Six months before, her BMR was +30 and thyroidectomy had been advised. She had refused surgery, but had taken intermittent rest cures, luminal, and iodine. Her weight had decreased gradually from 59 to 45 kg. Improvement in nervousness and sleep appeared after 3 weeks of PABA treatment, concurrent with a gradual gain in weight. After 9 months therapy, there was still some enlargement of the thyroid which had receded considerably. Nevertheless, the patient remained well and when seen again 6 months later, stated that she felt perfectly healthy and was working 8 hours daily as a secretary.

Case 5 (L.M.) was first seen in February, 1944. The patient stated that her illness had lasted about 8 months during which time she had lost 23 kg of weight, had attacks of palpitation and constant nervousness. She had been given daily injections of prostigmin in December, 1943, when her BMR had been found by a clinical laboratory to be +40. Subsequently, the palpitation and feeling of heart pounding had been somewhat relieved, but her general condition became even worse. Simultaneously with PABA therapy her weight increased and after 3 months the patient was able to go about her housework and to sleep well. At the end of 8 months treatment, the patient felt well and remained so for the following 4 months when she was last seen.

Table I summarizes the data obtained on the first 6 patients with thyrotoxicosis. PABA was always administered parenterally in form of its sodium salt and the effect of oral therapy is being determined. Seven additional hyperthyroid cases are at present under treatment. In none of the patients did untoward reactions ever occur, thus confirming the remarkably low toxicity of PABA observed in the treatment of typhus.[10]

It is not intended to offer any speculations as to the mode of action of PABA, but comparative experimental studies have been started with PABA, thiourea and thiouracil to demonstrate their respective roles in thyroid and pituitary activity. A change in intestinal flora, as a factor contributing to the beneficial results obtained, is a possibility in the light of Landy's analyses reported by Kornberg *et al.*[11] showing a 100-fold concentration of PABA in the cecum of rats treated parenterally with 50 mg of PABA per kg body weight, *i.e.*, approximately the same dosage given to the patients reported in the present communication.

Whatever the mode of action may be, PABA is a naturally occurring substance with a very favorable therapeutic index.[12] It was found to have definite beneficial effects in 6 cases of hyperthyroidism receiving its sodium salt parenterally.

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[10] Yeomans, A., Snyder, J. C., Murray, E. S., Zarfonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

[11] Kornberg, A., Daft, F. H., and Sebrell, W. H., *J. Biol. Chem.*, 1944, **155**, 193.

[12] Ansbacher, S., *Vitamins and Hormones*, 1944, **2**, 215.

14987

Influence of Cod Liver Oil on Deposition of Ceroid in the Nutritional Rat Cirrhosis.

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The production of cirrhosis of the liver in rats on a diet deficient in protein (Methionine) and free from choline, has been described by various investigators.[1,2,3,4] The cirrhosis is accompanied by the deposition of a peculiar insoluble pigment which has been given the name ceroid by Lillie and coworkers.[5] The staining properties of this pigment, which is found not only in the liver but also in lymph nodes, lungs, pancreas, spleen, bone marrow and adrenal cortex, have been extensively described by Endicott and Lillie.[6] Popper, György, and Goldblatt[7] found it to give a golden brown fluorescence which they thought to be very useful for its identification. An insoluble, acid-fast, ceroid-like substance was produced by Endicott[8] in rats by injection of cod liver and linseed oils. Endicott observed a similar substance in a human case of pneumonia due to aspiration of cod liver oil. Endicott, Daft, and Sebrell[9] have recently reported on the production of hepatic cirrhosis without ceroid in rats by feeding protein-deficient diets without cod liver oil. They concluded that a substance or substances present in cod liver oil cause the appearance of this pigment.

In similar experiments to be described, it was found that restriction of cod liver oil in the diet will lead to a marked reduction of ceroid in the experimental liver cirrhosis in rats. Complete omission of cod liver oil frequently leads to somewhat less severe cirrhosis, without any trace of ceroid in some cases, but with ceroid deposition in others. The use of peanut meal and especially of fibrin in combination with small amounts of cystine as protein source was found to produce marked cirrhosis after 90 days in nearly all surviving animals with very extensive ceroid deposition in those which received cod liver oil.

Experiments. (A) *Casein as Protein Source.* 30 white rats, weighing between 150 and 200 g, were placed on a diet composed of 67.8% sucrose, 20% hydrogenated cottonseed oil (Crisco), 8% casein (Smaco), 4% salt mixture No. 2 USP XII, and 0.2% cystine. They received in addition daily 20 μg of thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride and 100 μg of calcium pantothenate, and weekly 1 drop of cod liver oil.

The rats were killed after 120 days with the exception of 2 which died after 112 and 116 days respectively. Twelve animals showed marked to very extensive cirrhosis, 9 fatty infiltration with beginning cirrhosis, and the remaining animals showed varying degrees of fatty changes. In this latter group, no ceroid deposition was seen. Among the animals with beginning cirrhosis, 3 showed traces and 1 a moderate amount. Of the 12 livers with marked cirrhosis, only in 2 moderate amounts of ceroid were seen, while in the remaining ones only traces to very small amounts of

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[1] György, P., and Goldblatt, H., *J. Exp. Med.*, 1939, **70**, 185; 1942, **75**, 355; PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 492; György, P., *Am. J. Clin. Path.*, 1944, **14**, 67.

[2] Webster, G., *J. Clin. Invest.*, 1942, **21**, 385.

[3] Blumberg, H., and McCollum, E. V., *Science*, 1941, **93**, 598; Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

[4] Lillie, R. D., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1941, **56**, 1255.

[5] Lillie, R. D., Ashburn, L. L., Sebrell, W. H., Daft, F. S., and Lowry, J. V., *Pub. Health Rep.*, 1942, **57**, 502.

[6] Endicott, K. M., and Lillie, R. D., *Am. J. Path.*, 1944, **20**, 149.

[7] Popper, H., György, P., and Goldblatt, H., *Arch. Path.*, 1944, **37**, 101.

[8] Endicott, K. M., *Arch. Path.*, 1944, **37**, 49.

[9] Endicott, K. M., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 330.

ceroid could be detected. No ceroid was seen in one of these livers. In previous experiments, using a similar diet with 2% cod liver oil, much larger amounts of ceroid were seen in livers showing early or progressive cirrhosis.

(B) *Peanut-Casein Diet.* Eight male weaning rats, weighing between 35 and 45 g, were given a diet consisting of 51.7% saccharose, 30% peanut meal (prepared according to Engel and Salmon),[10] 6% casein (Smaco), 6% hydrogenated cottonseed oil (Crisco), 4% salt mixture No. 2 USP XII, 2% cod liver oil, and 0.3% cystine. The food contained in addition 10 mg of riboflavin, thiamine hydrochloride, and pyridoxine hydrochloride respectively and 50 mg of calcium pantothenate in 1000 g.

Three animals died in the beginning of the experiment with signs of acute choline deficiency. The remaining 5 animals were killed after 90 days. They were in good condition and weighed between 60 and 120 g. Four animals presented cirrhosis, 2 quite progressed. The liver of all these animals showed a large amount of ceroid and large mesenteric lymph nodes, containing much ceroid, were seen in 2 cases. In 4 instances degenerative changes were found in the kidneys.

A second group of 11 male weaning rats were given a similar diet without cod liver oil. These rats received instead 1 drop of cod liver oil each week. Six animals survived the first month. Early cirrhosis with traces of ceroid deposition were seen after 55 and 63 days respectively. Distinct cirrhosis and a fair amount of ceroid was seen in 2 other rats after 78 and 83 days respectively. Of 2 animals killed after 90 days, one showed a moderate cirrhosis with a moderate amount of ceroid, while very little ceroid was found in the other rat. Degenerative kidney changes of varying degree were found in all cases.

A third group of 8 male weaning rats was placed on a similar diet without cod liver oil. These animals received 3 times weekly 40 μg carotene (Smaco) dissolved in liquefied cotton seed oil. No vitamin D was given since this vitamin is not essential for rats.[11] Six animals lived longer than 30 days. One animal died after 49 days, showing beginning cirrhosis without ceroid. The remaining 5 were killed after 90 days. They were in good condition and weighed between 65 and 90 g. Two of the livers showed moderate cirrhosis. Both contained a small amount of ceroid. Five out of 6 animals showed focal degenerative kidney changes.

(C) *Fibrin as Protein Source.* A group of 12 male weaning rats, weighing between 35 and 45 g, were placed on a diet of the following composition: 77.3% saccharose, 10% blood fibrin (Difco), 6% hydrogenated cottonseed oil, 4% salt mixture No. 2 USP XII, 2% cod liver oil, and 0.3% cystine. In addition, this food contained 10 mg of riboflavin, thiamine hydrochloride, and pyridoxine hydrochloride respectively, and 50 mg of calcium pantothenate in 1000 g.

Eight animals survived longer than 30 days. One died after 30 and another after 48 days. One was sacrificed after 63 days. This latter showed beginning cirrhosis without ceroid deposition. The remaining 5 animals were killed after 90 days. They had not gained any weight, were in poor general condition, and showed focal alopecia. Four of these 5 animals revealed marked and 1 moderate cirrhosis. One animal showed ascites. Enlarged brownish mesenteric lymph nodes were seen in all 5 cases. On microscopic examination all 5 livers contained large amounts of ceroid (Fig. 1). Mesenteric lymph nodes and spleen contained the same pigment. All 8 animals showed degenerative changes in the kidneys.

A second group of 12 male weaning albino rats were kept on a similar diet without cod liver oil. The animals received 3 times weekly 40 μg of carotene, dissolved in liquefied Crisco. Eight animals lived longer than 30 days. In 2, which lived for 57 and 65 days respectively, a moderate cirrhosis but no ceroid deposition was seen. One animal showed beginning cirrhosis and a small amount of ceroid after 86 days. Of the 4 remaining animals which were killed after 90 days, one showed marked and 3 moderate cirrhosis.

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[10] Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

[11] Griffith, J. Q., and Farris, E. J., *The Rat in the Laboratory Investigation*, 1942, J. B. Lippincott Co., p. 97.

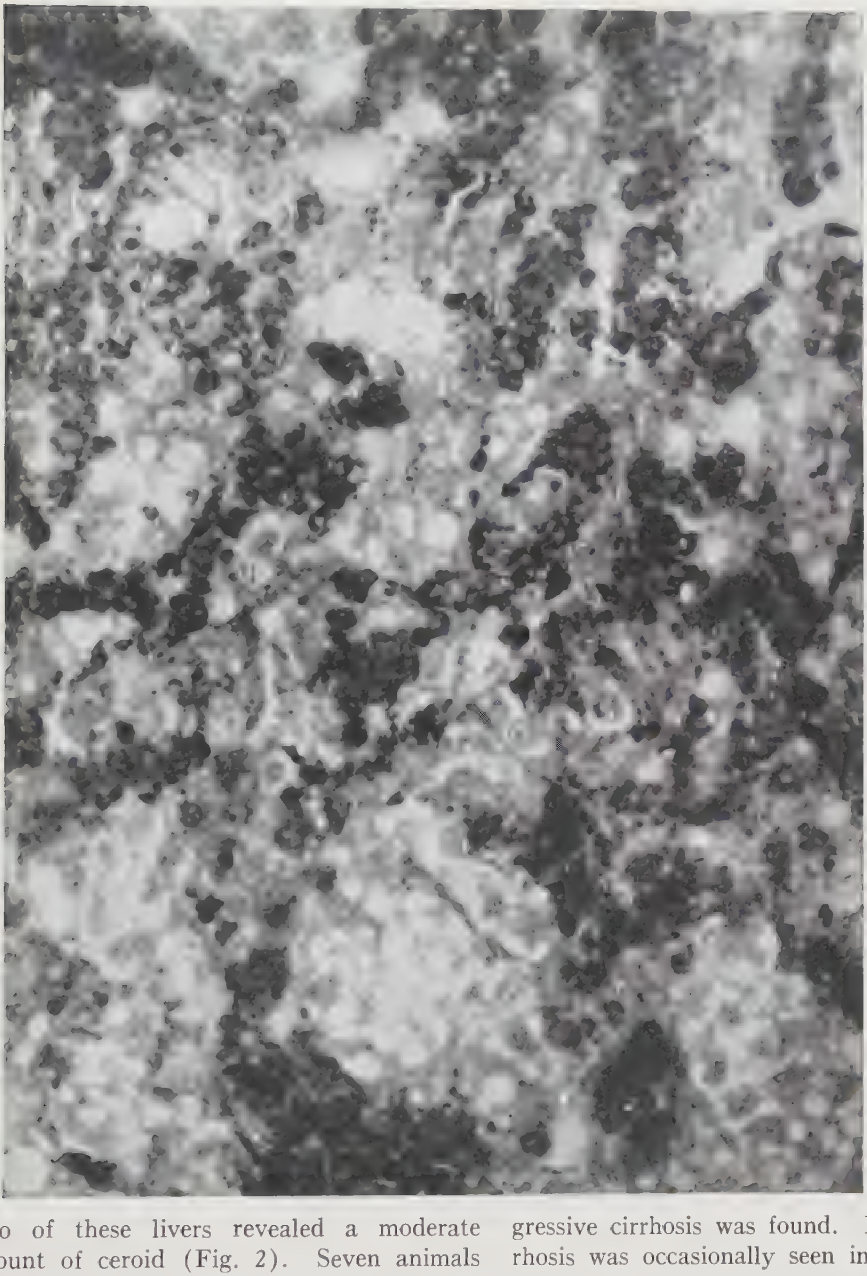

FIG. 1.
Liver showing extensive ceroid deposition in a rat after 90 days on a fibrin diet, containing 2% cod liver oil. Ziel-Neelson stain. × 150.

Two of these livers revealed a moderate amount of ceroid (Fig. 2). Seven animals showed varying degrees of degenerative kidney changes.

Comment. Extensive and progressive cirrhosis of the liver can be produced by feeding male weaning rats a choline-free diet in which the protein source is either 10% fibrin or 30% peanut meal plus 6% casein, and to which 0.3% cystine is added. In nearly all animals surviving 90 days, fatty infiltration and progressive cirrhosis was found. Beginning cirrhosis was occasionally seen in less than 60 days. The changes were most distinct on the fibrin diet. Very large amounts of ceroid were found under these experimental conditions. The microscopic appearance of the cirrhotic livers corresponded closely to that described by various investigators. The normal lobular architecture was destroyed and small and larger areas of liver tissue were separated by fibrous bands. Single as well as

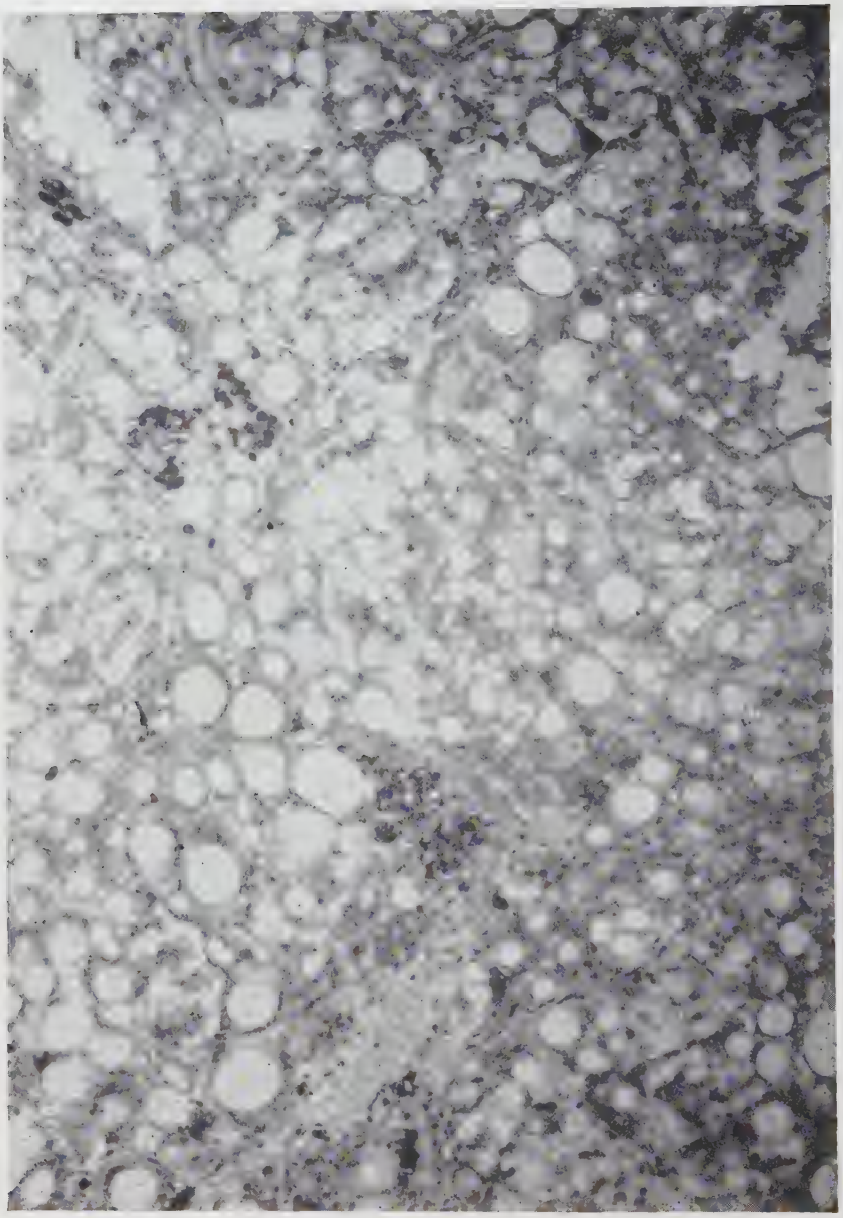

FIG. 2.
Liver showing moderate ceroid deposition in a rat after 90 days on a fibrin diet without cod liver oil. Ziel-Neelson stain. × 150.

islands of liver cells, some of them necrotic, were seen enclosed in the proliferating connective tissue. Many liver cells contained large amounts of fat.

A peanut-casein diet had been used by Engel[12] for the production of liver cirrhosis in rats. However, Engel kept his animals for 16 months on the experimental diets. Engel fed small amounts of choline in the beginning of the experiments, in order to avoid the detrimental influence of choline deficiency on the kidneys. While some animals died in the beginning of the experiments here reported, due to acute choline deficiency, a good number survived. Most of the surviving animals showed the typical degenerative kidney lesions found in choline deficiency.[13,14,15]

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[12] Engel, R. W., *Fed. Proc.*, 1943, **2**, 62.

Most investigators reported the occurrence of cirrhosis in rats only after a longer period of feeding the experimental diets. Daft, Sebrell, and Lillie[16] found, however, moderate to marked cirrhosis in weaning rats on a diet containing 4% casein and 0.5% cystine, after an average period of 83 days. In the experiments here reported cirrhosis can be produced in a short time even by giving large amounts of protein, deficient in methionine and enriched by cystine.

If the diet used in these experiments contained 2% cod liver oil, very large amounts of ceroid are deposited. If, however, the cod liver oil intake is restricted to 1 drop weekly, markedly less ceroid is formed. Hepatic cirrhosis is also observed in rats which are fed the experimental diets without any cod liver oil. However, the cirrhosis seems less severe in the majority of cases. In some instances, no ceroid was found in these cirrhotic livers, thus confirming the results of Endicott and co-workers. In other cases, however, ceroid deposition occurred, although in much smaller amounts than in the controls receiving cod liver oil. Cod liver oil can, therefore, not be the only reason for the occurrence of ceroid.

So far ceroid has only been observed in the livers of rats fed a choline-free, protein-deficient diet. It has, however also been found in the livers of mice in which cirrhotic changes had been produced by means of carbon tetrachloride[17] and O-aminoazotoluene.[18] These animals were on a full normal diet without added cod liver oil.

Summary. Regular and severe liver cirrhosis can be induced in male weaning rats on a choline-free, protein-deficient diet after 90 days, if the protein source is either fibrin or peanut meal plus casein. Early cirrhosis is occasionally observed in less than 60 days. Very large amounts of ceroid are deposited by the use of these diets, containing 2% cod liver oil. The amount of the deposited ceroid is markedly reduced if the intake of cod liver oil is restricted to one drop weekly. On the same diet without any cod liver oil, the produced cirrhosis is less severe. Some of these livers contain no ceroid, others small amounts of this pigment. Cod liver oil is therefore not the only cause for ceroid pigmentation in nutritional cirrhosis. It enhances, however, its formation very markedly.

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[13] György, P., and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

[14] Christensen, K., *J. Biol. Chem.*, 1940, **133**, xx; *Arch. Path.*, 1942, **34**, 633.

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[16] Daft, F. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 228.

[17] Edwards, J. E., and Dalton, A. A. J., *J. Nat. Cancer Inst.*, 1942, **3**, 19.

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14988

Serial Peritoneoscopic Examinations as a Means of Studying Experimental Liver Damage in Dogs.*

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Most experimental observations of the histological changes occurring in the liver as the result of the administration of toxic agents have been based upon postmortem findings. Such a method necessitates either sacrifice of the animal or waiting until the effects of the agent are lethal.

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† Santiago, Chile, S.A. Traveling Fellow, Rockefeller Foundation.

Experiments designed to study the degree of liver protection afforded by various diets for dogs receiving repeated doses of carbon tetrachloride have been under way in this laboratory for the past 14 months. Methods that would permit inspection and biopsy of the livers of these animals at intervals were desired. Many investigators have secured liver biopsies in dogs during the course of experimentally induced damage by performing a laparotomy. This has certain disadvantages. The use of a general anesthetic agent is necessary, and the number of abdominal incisions that can be performed is limited. Of more importance, however, is the intolerance for major surgical procedures of animals suffering from induced liver damage. Davis and Whipple[1] observed that even aseptic precautions did not prevent postoperative infections and that the incisions in such animals had a tendency to open superficially. Bollman and Mann[2] stated that many of their dogs subjected to carbon tetrachloride exposure would "die following excision of a small piece of liver for histological examination, a procedure that is relatively harmless to the normal animal." We have been gratified to observe in the course of such experiments that dogs tolerate repeated peritoneoscopic examinations and liver biopsy very well. This is a minor surgical procedure and can be performed with local anesthesia.

Peritoneoscopy is of considerable value in the clinical study of liver disease. Benedict[3] has suggested the use of peritoneoscopy in patients with cirrhosis to observe the progression or regression of the process and evaluate therapeutic procedures. So far as is known the method has not been widely employed in the experimental laboratory. It is of interest to note that Kelling, a German surgeon, first introduced the method to the 73rd Congress of German Naturalists and Physicians in 1901, by demonstrating a living dog into whose abdomen he had inserted a cystoscope for the purpose of examining the viscera. The procedure is said to have been used in the Veterinary Institute of Dresden before 1910 in certain physiological experiments (Nadeau and Kampmeier[4]). Horan[5] recommended preliminary trials of the method in dogs as a means of gaining familiarity with the instrument before clinical use.

Method. The instrument used is the Ruddock peritoneoscope;[‡] the procedure as recommended by Ruddock[6] for clinical purposes has been followed. A preliminary sedative dose of morphine (5 mg per kilo of body weight) is given to the dog by hypodermic one hour prior to the examination. The animal is strapped to an operating table, the abdomen is shaved, scrubbed and prepared with a suitable antiseptic agent. The abdomen is draped with sterile towels and a laparotomy sheet. Sterile technic is used throughout the operative procedure. Sterilization of the peritoneoscope and its accessory parts is carried out for 20 minutes in a mercury oxycyanide solution of 1-500 concentration. All instruments are rinsed in sterile water before transfer to the instrument table. The point chosen for insertion of the instrument is the midline, one-third the distance from the xiphoid to the symphysis. Infiltration of 1% procaine hydrochloride in the skin and underlying tissues produces satisfactory local anesthesia. Care is taken to see that a small portion (2 cc) is infiltrated just at the parietal peritoneal surface. A small skin incision of 5 mm suffices for introduction of the blunt pneumoperitoneum needle. Air is pumped into the peritoneal cavity until the abdomen is tense. This is accomplished by means of a small hand bulb, room air being used. Filtration through cotton is unnecessary; the amount of air introduced is not important. The initial skin incision is enlarged to 1 cm and the larger trocar is inserted into which the peritoneoscope itself fits snugly.

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[1] Davis, N. C., and Whipple, G. H., *Arch. Int. Med.*, 1919, **23**, 711.

[2] Bollman, J. L., and Mann, F. C., *Ann. Int. Med.*, 1931, **5**, 699.

[3] Benedict, E. B., *New England J. Med.*, 1944, **230**, 125.

[4] Nadeau, O. E., and Kampmeier, O. F., *S. G. and O.*, 1925, **41**, 259.

[5] Horan, T. N., *Mich. St. Med. Soc. J.*, 1937, **36**, 634.

[‡] American Cystoscope Makers, Inc., New York, N.Y.

[6] Ruddock, J. C., *Western J. Surg.*, 1934, **42**, 392.

The presence of pneumoperitoneum or the manipulation of the instrument causes the animal no apparent discomfort. Inspection of the peritoneal cavity can readily be carried out by the illumination furnished by the small bulb just distal to the lens. Due to the anatomical arrangement in the dog a more complete inspection of the liver and spleen can be accomplished than is true in the human subject. Specimens of liver tissue can be secured by means of a biopsy forceps. The tissue specimen that can be secured is a triangular shaped block measuring about 5 mm in length and 4 mm at the base. This represents 40 to 50 mg of liver tissue. Specimens can be secured from various lobes as desired. Serious hemorrhage from the biopsy site has not been encountered; animals having severe liver damage as judged by the histological and gross appearance and altered liver function tests exhibited only minimal bleeding. Cautery can be employed; we have used it occasionally, more to gain familiarity with the maneuver than to control hemorrhage. Application of a thrombin solution§ to the biopsy site and application of small amounts of a soluble cellulose substance (Oxycel§) have recently been tried; again this has been done as a preliminary to future clinical trial rather than as a necessity for the control of hemorrhage from the dog's liver. Upon completion of the observation, the air is released from the abdominal cavity and the trocar is removed. The skin edge is closed by means of a single Michael clip which is left in place 5 days. No other closure is made. The animal, when released from the operating table, exhibits no ill effects and scampers about the room. Feeding schedules are resumed immediately postoperatively. Herniation of a piece of omentum at the site of the 1 cm long incision was observed in but one animal, this occurred after the seventh examination. Recovery was uneventful and did not interfere with further examination in this same animal.

Material. The first series of experiments in which this method was employed concerned a correlation study of the histology of the liver and the results of multiple liver function tests performed on the day that the biopsy was secured. In a group of 18 adult dogs, 145 peritoneoscopic examinations and liver biopsies were carried out in a 10-month period; 8 of the group were subjected to peritoneoscopy 10 or more times. Carbon tetrachloride was administered repeatedly to 15 of the animals. Three were maintained on a high-fat, low-protein diet. In a second series 9 animals receiving carbon tetrachloride have been subjected to 36 peritoneoscopic examinations and liver biopsy in a 2-month period. During each peritoneoscopic examination 3 or more biopsies were taken from different lobes of the liver.

Results. In no instance has a fatality occurred as a direct or indirect result of the procedure. The occurrence of hepatic necrosis and the development of nodular cirrhosis can be detected grossly by this method of observation. The validity of the observed gross appearances has been checked by subsequent autopsies.

In Fig. 1, representative photomicrographs of serial liver biopsies in 3 of the animals are shown. Dog No. 17 (Fig. 1 A, B, and C) was maintained on a high-fat, low-protein diet and received repeated doses of carbon tetrachloride via stomach tube. Fig. 1-A represents the histological appearance prior to the administration of the toxic agent; 1-B represents the third biopsy taken 49 days later after 39 cc of carbon tetrachloride in divided doses had been administered. Fig. 1-C represents the fifth biopsy taken on the 93rd day of the experiment at which time a total of 111 cc of carbon tetrachloride had been given.

In Fig. 1-D, E and F the photomicrographs represent biopsies secured in dog No. 5, maintained on a high-protein, low-fat diet. Fig. 1-D is the appearance at the start of the experiment and 1-E the third biopsy taken 83 days later by which time 91 cc of carbon tetrachloride had been given in divided doses. In Fig. 1-F the fifth biopsy is shown taken on the 125th day by which time 239 cc of carbon tetrachloride had been administered. Serial biopsies from Dog 3 are shown in Fig. 1-G, H, and I. This animal was maintained on a high-fat diet, no toxic agent had been given. Photomicrographs shown represent the control liver biopsy, Fig. 1-G, the fourth biopsy,

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§ Parke, Davis and Co., Detroit, Michigan.

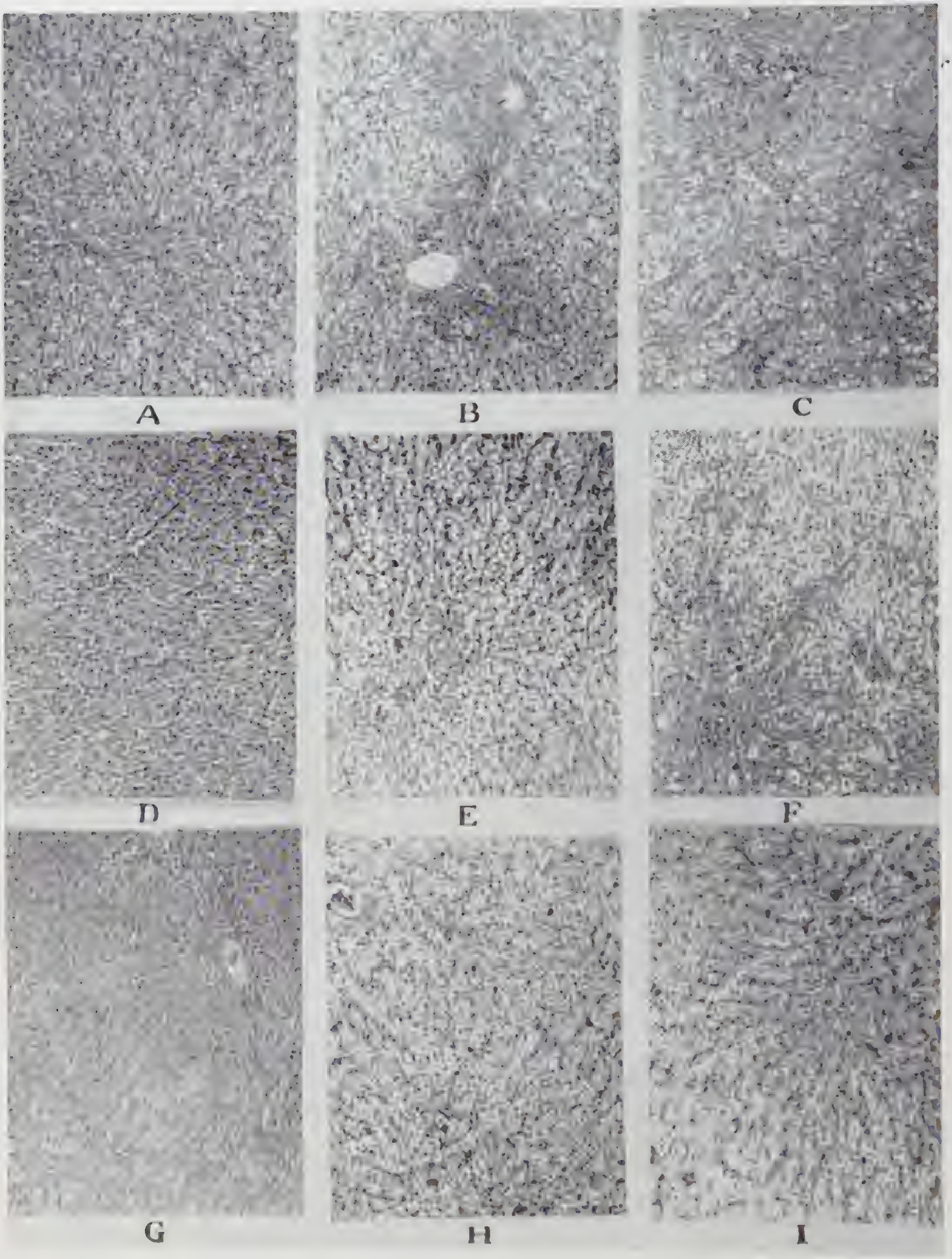

FIG. 1.
Photomicrographs of liver biopsies secured at intervals by means of repeated peritoneoscopic examination. For details see text.

Fig. 1-H taken on the 104th day, and Fig. 1-I the eighth biopsy taken on the 227th day.

Summary and Conclusions. 1. The procedure of peritoneoscopy, as described by Ruddock, is a satisfactory means of inspecting the liver and of securing liver biopsy specimens in the dog. The examination can be accomplished without pain to the animal using local anesthesia, after morphine sedation. Repeated examinations with inspection and biopsy of the liver can be made over a period of time in the same dog.

2. A series of 145 peritoneoscopic examinations in a group of 18 dogs with toxic or dietary liver damage was carried out in a 10-month period. Eight dogs in this group were subjected to peritoneoscopy 10 or more times. A second similar group of 9 dogs were subjected to 36 such examinations in a 2-month period. In each instance multiple liver biopsies were taken.

3. No fatalities attributable to this procedure have occurred even when performed on dogs exhibiting severe liver damage.

14989

The Intracranial Toxicity of Influenza Virus for Mice.*

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A study of the properties of the influenza A virus, designated IC strain, isolated during the 1943 epidemic at Iowa City, involved the injection of the virus into the brains of Swiss mice to determine its neurotropic qualities. Two strains of influenza virus are known to be neurotropic, one reported by Stuart-Harris[1] and the other by Francis and Moore.[2] These strains propagated in the mouse brain and after several passages produced convulsions and death. Henle and Henle[3] working with non-neurotropic strains showed that active virus injected in sufficient concentrations produced convulsions but they did not obtain evidence of virus propagation. Although the investigation herein reported was completed before the publication of Henle and Henle[3] this work will serve as a confirmation of their experiment. Evans and Rickard[4] found that the injection of type A and type B influenza virus in the rabbit's eye produced corneal opacity without multiplication of the virus. Rake and Jones[5] using viruses of lymphogranuloma venereum, mouse pneumonitis, and meningo-pneumonitis have shown that these viruses exert a toxic effect.

All virus and antibody titers given in this report were determined by the Hirst[6] hemagglutination test as modified by Salk.[7]

Preliminary experiments indicated that allantoic fluid containing the IC strain of influenza virus, injected in 0.03 ml doses intracranially in mice, resulted in convulsions in a majority of cases after 48 hours, with death occurring on the second or third day, rarely as late as the fifth. The mortality rate was 50-100% depending upon the concentration of the virus. That the convulsions were not caused by bacterial infection was demonstrated

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* This investigation was aided in part through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

[1] Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.

[2] Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, **72**, 717.

[3] Henle, G., and Henle, W., *Science*, 1944, **100**, 410.

[4] Evans, C. A., and Rickard, E. R., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 73.

[5] Rake, G., and Jones, H. T., *J. Exp. Med.*, 1944, **79**, 463.

[6] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[7] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE I.
The Effect of Dilution and Heat Inactivation on the Convulsive Factor.

Material injected	No. of mice injected	No. developing convulsions
Undiluted IC virus in allantoic fluid	10	8
Same, heated at 56°C	10	0
IC virus in allantoic fluid diluted 1:10	10	0
Undiluted normal allantoic fluid	10	0

by failure to isolate bacteria from the brain by both aerobic and anaerobic cultural methods. Although the virus could be recovered from the brain at the time of convulsions, it was present in very small quantities. However, all attempts to passage from brain to brain of mice were uniformly unsuccessful. In no case could the virus be recovered from the second passage by inoculation of brain material into the allantoic sac of chick embryos.

Since there was no evidence of propagation on the part of the IC influenza strain in the mouse brain, it seemed reasonable to think that other non-neurotropic strains would give the same results. Therefore, an experiment using the PR8 influenza virus was tried. The same symptoms and results were produced. Mouse brains removed 48 hours after intracranial inoculation and at the time of convulsions, were injected into the allantoic sac of embryonated eggs. The virus was isolated in some, but not all instances, again giving evidence that little, if any, propagation took place in the mouse brain.

The Lee strain of influenza B virus was tested in the same manner and no difficulty was encountered in duplicating the above results. No attempt was made to recover this virus from the brain at the time of convulsions.

Since there was no evidence of propagation of virus in the brain it was decided to determine if an inactive virus would give these neurological symptoms. Also included in this experiment was the effect of dilution on the convulsive factor. A control of normal allantoic fluid was added. The IC strain was grown in the allantoic sac of embryonated eggs. The fluid was removed and pooled from several embryos after 48 hours incubation at 37°C and was found to have a hemagglutination titer of 1:3,200. Intracranial injections of 0.03 ml of undiluted infected allantoic fluid were made into the brains of month-old Swiss mice and a similar group was inoculated with an equal volume of 1:10 dilution of the virus-containing material. Another group of mice received this volume of undiluted virus-containing fluid which had been heated at 56°C for 45 minutes. The control mice were given undiluted normal allantoic fluid in the same manner. The results are shown in Table I.

Table I shows that the undiluted, unheated material produced convulsions while the heated and the diluted allantoic fluid failed to elicit such reactions. The normal fluid had no effect.

Because propagation of the influenza virus in the brains of mice seemed unnecessary for the production of convulsions, it was thought that these symptoms might be caused by the toxicity of the virus particle itself, or by some soluble substance that was not absorbed and eluted from the chicken red blood cells as was the virus. With this in mind, an experiment was planned to test these possibilities, and also to determine whether the convulsive factor passed an unglazed porcelain filter. Included in the test was the effect of inactivation by heat on the concentrated virus.

The PR8 strain of influenza virus was used after growth in the allantoic sacs of embryonated eggs. After 48 hours incubation at 37°C the pooled fluid having a hemagglutination titer of 1:12,800 was tested for sterility and during the sterility test the allantoic fluid containing virus was stored on dry ice. After proving that the fluid was free from bacteria, an equal volume of 3% sterile suspension of chicken red blood cells was added to a portion. This mixture was incubated for 2 hours at 4°C with occasional gentle shaking, centrifuged in the cold, the supernatant fluid re-

TABLE II.
The Association of the Convulsive Factor with the Virus Particle.

Material injected	Amount intracranially	No. of mice injected	No. developing convulsions
Unfiltered concentrated virus; titer 1:102,400	0.03 ml	10	10
Conc. virus; titer after filtration 1:51,200	0.03 ml	10	10
Supernatant fluid after 1 absorption with equal volume 3% sterile red blood cells	0.03 ml	10	0
Conc. virus; titer 1:102,400; heat inactivated at 56°C for 45 min	0.03 ml	10	0

moved, and .9% sterile saline added to the sedimented red blood cells in such amount that the virus was concentrated ten times after elution. The virus was eluted by a 2-hour incubation at 37°C and after removal of the red blood cells the titer was found to be 1:102,400. Eight ml of the concentrated virus suspension was filtered through a Coor's porcelain filter of 3 porosity. The hemagglutination titer of the filtrate was 1:51,200. Thus a substantial amount of the virus was removed by filtration.

Swiss mice, 5 weeks old, were injected intracranially with 0.03 ml each of the following materials: Concentrated virus with a titer of 1:102,400; filtrate of concentrated virus with a titer of 1:51,200; the supernatant fluid after one absorption with an equal volume of 3% sterile red blood cells; and concentrated virus inactivated at 56°C for 45 minutes. The results of this experiment are given in Table II.

It is obvious that the convulsive factor was absorbed on and eluted from the red blood cells. It did not remain in the supernatant fluid after one absorption in sufficient concentration to produce symptoms. The convulsive factor was not removed by filtration through unglazed porcelain, and inactivation at 56°C wholly destroyed the ability of the concentrated virus to produce convulsions.

Another experiment was devised to determine whether or not the convulsive factor was specifically neutralized by immune serum. For this test sterile undiluted allantoic fluid containing PR8 influenza virus, having a hemagglutination titer of 1:6,400, was mixed with sterile anti-influenza A and anti-influenza B immune mouse serum. The anti-hemagglutination titer of the anti-A serum was 1:5,120 and that of the anti-B 1:81,920. The following mixtures were made:

TABLE III.
Specific Neutralization of the Convulsive Factor by Antiserum.

Material injected	No. of mice	No. developing convulsions
A antiserum + A virus	10	0
B antiserum + A virus	10	6
Saline + A virus	10	8

0.4 ml allantoic fluid containing influenza A + 0.06 ml anti-A mouse serum.

0.4 ml allantoic fluid containing influenza A + 0.06 ml anti-B mouse serum.

0.4 ml allantoic fluid containing influenza A + 0.06 ml saline (control).

Swiss mice, 21 days old, were injected intracranially with 0.03 ml each of the above mixtures and the results are shown in Table III.

It is evident that influenza A virus neutralized with specific serum was unable to cause convulsions but that the anti-B serum gave no protection against the convulsive factor.

The results of these experiments demonstrated that the convulsive factor is closely associated with the virus particle and that it is absorbed and eluted by red blood cells as is the case of the virus. The convulsive factor is heat labile. No evidence was found that the virus propagated in the mouse brain, therefore the most logical conclusion would be that the virus protoplasm was the toxic substance in a manner similar to the endotoxins of bacteria. However, until some form of inactive virus is found that will produce neurological symptoms, one should have some hesitation in concluding that the convulsions were caused by a toxicity in the manner of bacterial endotoxins.

Summary. Although incapable of propagating in the mouse brain, the influenza virus strains used in these experiments produced convulsions in this species when injected intracranially. The convulsive factor was absorbed on and eluted from chicken red blood cells as was the virus. The convulsive factor was inactivated by heating at 56°C for 30 minutes. It was specifically neutralized by antiserum.

14990

Simultaneous Infection in Laboratory Animals with Murine and Classic Typhus. II. Recovery of Strains.*

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In a previous paper[1] we reported on the production of typhus pneumonia in rats and mice with a mixture of murine and classic rickettsiæ. The assumption that both viruses were present was supported by serological and immunological studies. The experiments here reported demonstrate that the mixed infection was still detectable after 10 generations in the lungs of mice and that then both strains were recovered by inoculation in guinea pigs. The means by which we could separate the murine from the classic strain were based on the observation of the behavior of male and female guinea pigs towards the 2 types of typhus infection. Male guinea pigs, when inoculated with murine typhus, develop, as is well known, a characteristic swelling of the scrotum accompanied by a rise of temperature which lasts several days. The same virus inoculated into female guinea pigs produces a considerably milder disease apparent only in a smaller rise of temperature for a shorter period than in male animals. Often the disease is inapparent. The transfer of the strain from female to female is difficult and the strain is often lost after 2 or 3 generations. On the other hand, females react readily to classic typhus infection and the strain can be preserved indefinitely.

Material and Methods. The murine typhus strain used was isolated from a case of human typhus in 1936 in Mexico City. It has been kept by a considerable number of transfers in guinea pigs and constantly produces scrotal swelling in males. In females inoculated with tunica material the infection is restricted to less than 4 days of moderate febrile reaction. The classic virus is the "Breinl" strain which produces a febrile reaction lasting from 6 to 10 days in male and female guinea pigs when inoculated with 0.1 g of brain of an animal killed on the 5th day of fever.

The material for the inoculation of mice by the intranasal route was prepared according to methods described elsewhere.[2] Once the virus was adapted to the mouse lung, a mixture of equal amounts of murine and classic material was inoculated into mice by the intranasal route. At 48-hour intervals transfers were made using sulfathiazole[3] as a preservative to prevent contamination of the lungs by secondary invaders.

The separation of the 2 typhus rickettsiæ was made from the 10th transfer of the mixed virus from lung to lung. Guinea pigs, male and female, were inoculated by intraperitoneal route with an emulsion of an infected mouse lung. The material was diluted conveniently to reduce the chances of peritonitis by secondary invaders. Subsequent transfers from male to male guinea pigs were made using as inoculum the tunica vaginalis, while in female guinea pigs the inoculum was brain emulsion. Transfers from tunica material to female

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TABLE I.
Transfer of Mixed Typhus Virus from the 10th Generation in Mice into a Series of Male Guinea Pigs, Then Through a Series of Female Guinea Pigs and finally Through Males Again. Recovery of the Murine Strain.*

Transfer No.	Guinea pig No.	Material used for transfer	Days of fever	Scrotal swelling	Complement murine	Fixation classic	Day of bleeding
First Group.							
1	1 male	Mouse lung	13	Yes	640	320	22
2	3 ”	Tunica	K.f.t.	”	20	20	6
3	4 ”	”	9	”	640	160	22
4	6 ”	”	K.f.t.	”	5	0	6
5	7 ”	”	3	”	320	40	20
6	9 ”	”	2	No	320	20	21
7	10 ”	”	4	Yes	160	40	20
8	12 ”	”	4	”	320	80	30
	13 ”	”	4	”	80	40	30
9	15 ”	”	4	”	160	40	23
10†	17 ”	”	4	”	320	40	23
	18 female	”	K.f.t.	—	10	5	9
Second Group.							
2	19 ”	Brain	K.f.t.		160	0	15
	20 ”	”	0		0	0	22
3	21 ”	”	K.f.t.		0	0	9
	22 ”	”	7		320	20	16
4	24 ”	”	7		640	160	21
5‡	26 male	”	K.f.t.				
Third Group.							
2	28 ”	Tunica	2	Yes	80	5	20
3	30 ”	”	7	”	160	5	27

* The animals used for transfer of the strain are not included, except those bled for complement fixation tests.

† Series carried to the 19th transfer, then discontinued.

‡ Series carried to the 7th transfer.

K.f.t. means killed for transfer.

The figures in the Complement Fixation column indicate the maximum dilution of the serum producing a reaction with murine and classic typhus rickettsiæ.

guinea pigs were made whenever it was desired to study the reaction of females to the virus carried in males. Also brain material from the female group was transferred to males at various stages of the experiment to find out if the murine virus persisted in such a group.

Results. *Series of male guinea pigs.* Guinea pig No. 1 (Table I) showed a rather severe infection with 13 days of fever and swelling of the scrotum. Serial transfers to male guinea pigs starting from a mate of guinea pig No. 1 produced infection of less severity. The transfers were made usually to 2 animals one of which was used to obtain the tunica vaginalis for inoculation, the other animals being saved to be bled for serological typing of the infection. The virus produced scrotal swelling in all but 2 male guinea pigs. The experiment was discontinued after 19 transfers. At the 10th transfer the virus was inoculated to 1 female guinea pig and carried on through 8 generations in females. The infection was mild at the beginning and even failed to infect 1 animal. Subsequent transfers showed a certain increase in virulence through 5 generations. However, the infection became inapparent and was discontinued. Transfers from female guinea pigs carrying the "male strain" into the male guinea pigs produced scrotal swelling.

The serological study of this group of animals, including the females, showed a constant tendency to fix complement to a higher titer with the murine antigen. The diagnosis of murine type was corroborated by agglutination tests made with suitable antigens.

According to the clinical and serological results in this group of male guinea pigs it is apparent that after a few generations the murine virus remained alone. It is interesting to note that in early transfers, though complement fixation was higher in titer with the murine antigen, there was also a cross-fixation with classic rickettsiæ which was of greater

TABLE II.
Transfer of Mixed Typhus Virus from the 10th Generation in Mice into a Series of Female Guinea Pigs, Then through a Series of Males. Recovery of the Classic Strain.*

Transfer No.	Guinea pig No.	Material used for transfer	Days of fever	Scrotal swelling	Complement fixation murine	Complement fixation classic	Day of bleeding
First Group.							
1	1 female	Mouse lung	K.f.t.				
2	2 ,,	Brain	K.f.t.		0	5	9
3	3 ,,	,,	K.f.t.		0	0	9
4	4 ,,	,,	3		0	160	18
5	5 ,,	,,	7		5	160	21
6†	7 ,,	,,	8		0	320	20
	8 male	,,	K.f.t.	no	0	80	13
Second Group.							
2	24 ,,	,,	K.f.t.	,,	0	5	13
3	25 ,,	,,	K.f.t.	,,	0	0	10
4	26 ,,	,,	K.f.t.	,,	0	0	19
5	28 ,,	,,	8	,,	0	40	19
6	30 ,,	,,	5	,,	0	40	10
7	32 ,,	,,	9	,,	0	80	18

* Most of the animals killed for transfer of the strain not used for complement fixation tests are not included in this table.

† From the 7th to the 13th generation in female guinea pigs the bleedings made about the 20th day after the inoculation produced complement fixation tests ranging from 1:40 to 1:320 with the classic antigen, being lower or negative with murine rickettsiæ.

intensity than in subsequent transfers.

Female series. Female guinea pig 1 inoculated with lung material showed a rather moderate infection. Serial transfers were made with the brains of the animals killed usually from the 9th to the 10th day after the inoculation. Bleedings for complement fixation tests were made from 9 to 21 days after the inoculation. After the 5th generation the series was subdivided into female and male groups which were carried on for 7 to 8 transfers and then discontinued.

It is interesting to note that none of the 10 male guinea pigs carrying the "female strain" developed scrotal swelling. Serological studies in the female groups and the male subgroup showed that the type of the infection was classic typhus.

From these experiments it is apparent that the mixture of murine and classic typhus can be kept through an indefinite number of transfers in mice by intranasal inoculation of "lung material" and that the individual viruses can be recovered by means of inoculation into groups of male and female guinea pigs.

Summary. When mice are inoculated by the intranasal route with mixtures of murine and classic typhus the strains are carried on for an indefinite number of transfers and then both viruses can be isolated by inoculation into series of male and female guinea pigs. Serial transfers in both sexes seem to favor the prevalence of one or the other virus. Males favor the isolation of the murine type while females favor the preservation of classic typhus.

14991

A Method for Staining *Rickettsia orientalis* in Yolk Sac and Other Smear Preparations.*

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Ordinary dyes color rickettsiæ so faintly that special technics are employed to stain these microörganisms and to differentiate them from tissue elements. Satisfactory staining of rickettsiæ of endemic and epidemic typhus fever may be accomplished by means of a number of different staining technics,[1] including the Macchiavello, Castaneda, and Giemsa methods. In contrast, however, when these same techniques are applied to *Rickettsia orientalis,* the causative agent of scrub typhus fever (tsutsugamushi disease, mite-borne typhus), this microörganism stains poorly, if at all, in yolk-sac preparations. For this reason it is often difficult to distinguish suspected rickettsiæ from yolk granules and tissue particles in the background.

Because of the need for a reliable method of staining *R. orientalis* in yolk-sac smears, a study of staining technics and fixatives applied to this rickettsia was undertaken. It appeared possible that preliminary treatment of smears of infected yolk-sacs with lipid solvents might enhance the permeability of the microörganism to dyes. Accordingly, mixtures of chloroform and absolute alcohol, or of chloroform, absolute alcohol, and acetic acid, were employed for the preliminary treatment of smears, which were then stained by Giemsa's method. Such treatment was found to yield satisfactory results, and the details of the procedure are the subject of the present report.

Materials and Method. Fixative. Chloroform and absolute ethyl alcohol, with or without acetic acid, can be used in any one of the following proportions: (a) 1 part chloroform and 2 parts absolute ethyl alcohol, (b) 2 parts chloroform, 2 parts absolute ethyl alcohol, and 1 part glacial acetic acid, or (c) 3 parts chloroform, 6 parts absolute ethyl alcohol, and 1 part glacial acetic acid. The latter mixture, which constitutes Carnoy's fixative,[2] was found most satisfactory for routine use.

Stain. Giemsa stain is prepared in the following manner. To 39 ml distilled water are added 4 drops of 0.5% sodium bicarbonate and 1 ml of concentrated Giemsa stain (Grübler).

Procedure. Yolk-sac smears are prepared in the usual way and allowed to dry thoroughly. Cleaner preparations result when the yolk-sac is washed in 0.85% NaCl. The slide is then immersed in fixative for 15-30 minutes after which it is dried in a current of air, and placed in Giemsa stain for 15-30 minutes. It is then removed, dried and examined.

Results. When the foregoing method of staining was employed with smears of infected yolk-sacs, innumerable small, bluish-purple, well-defined bodies were seen which had the characteristic bipolar form of *R. orientalis* (Plate 1). These were usually extracellular, but a few densely packed intracellular rickettsiæ were also seen in some preparations. In smears of the same material which were not treated with lipid solvents before staining, only a few rickettsiæ could be seen, and these were neither sharply stained nor easily distinguishable from the background.

Smears of peritoneal fluid from mice or hamsters infected with *R. orientalis* were stained by this procedure. Well-defined rickettsiæ were seen intra- and extra-cellularly, in

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* The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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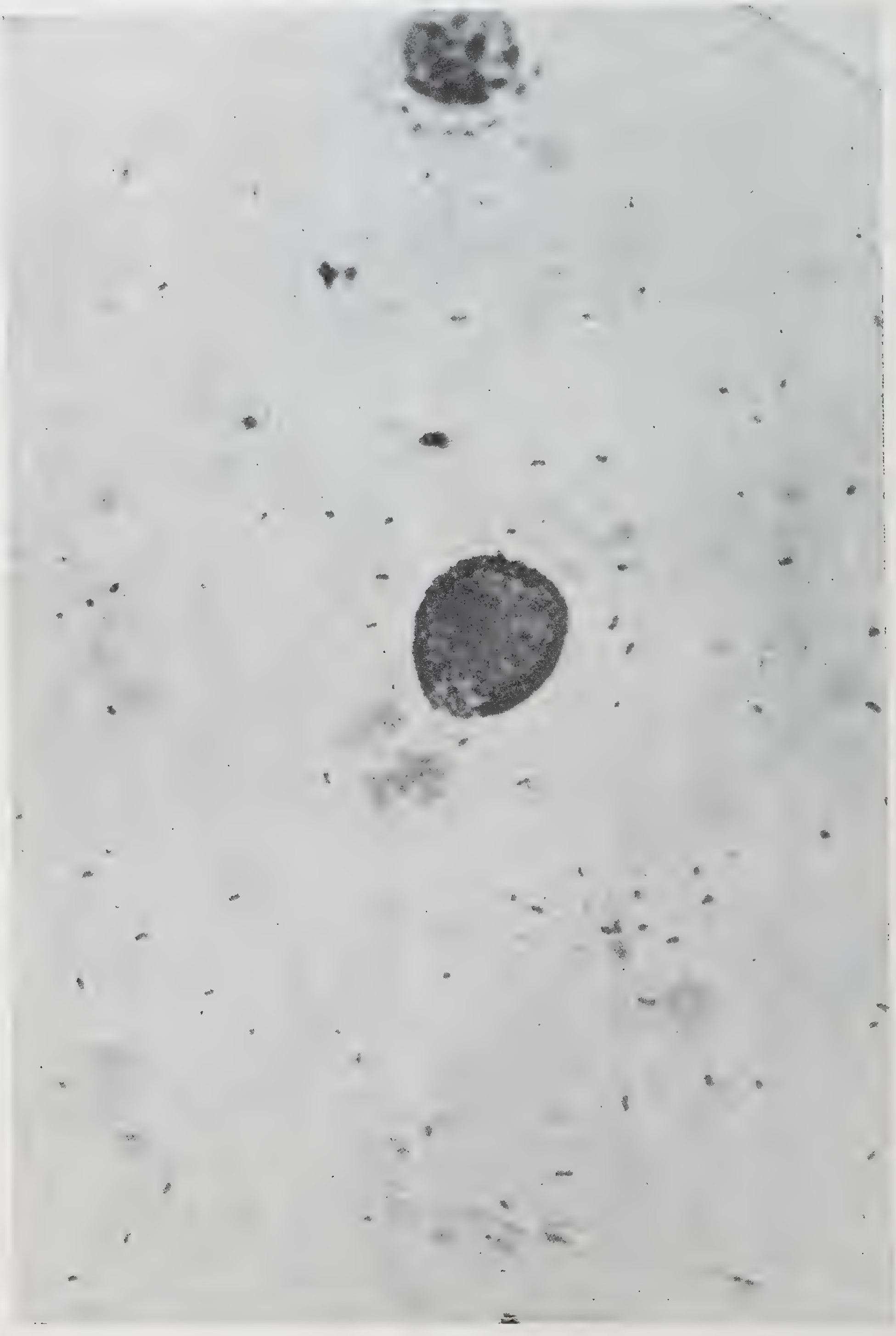

PLATE 1.
R. orientalis in smear of infected yolk-sac. Giemsa stain. × 2000.

larger numbers than in untreated preparations stained by the Giemsa method.

Summary. A method for staining *R. orientalis* in yolk-sac smears is described which yields uniformly satisfactory results. The efficacy of the method apparently depends upon the preliminary treatment of smears of infected tissue with lipid solvents.

14992

Dental Caries in the Cotton Rat. IV. Inhibitory Effect of Fluorine Additions to the Ration.*

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Miller[1] and several other workers have observed the inhibitory effect of fluorine upon the type of dental caries induced in the white rat by a coarse particle diet. Studies on the role of fluorine in the prevention of tooth decay have received increasing attention in the past few years. The cotton rat is very susceptible to the development of carious lesions when maintained on a ration containing sucrose or other soluble sugars.[2,3,4] Therefore it is fundamentally important to know the relation of fluorine in the ration to the production of this type of carious lesion.

Experimental. Fluorine as sodium fluoride (reagent grade) was added to the basal ration, 802 + 4% 1:20 liver extract, at levels ranging from 22.5 to 450 p.p.m. In these studies, the identical experimental procedure described by Schweigert *et al.*[4] was followed, with special attention to the division of litters between experimental and control groups. At the end of the 14-week experimental period after weaning, the cotton rats were sacrificed and the heads prepared for the evaluation of the incidence and extent of carious lesions. In addition to the caries evaluation, the teeth were examined for evidence of any toxic effect of the dietary fluorine.

When dextrin is substituted for all the sucrose in the basal ration, the incidence of carious lesions is greatly reduced.[2,3,4] Therefore, analyses of the dextrin and sucrose were made to determine their fluorine content. In this way it was possible to decide whether the inhibitory effect of dextrin upon the formation of carious lesions might be due to its fluorine content. The ashing of the samples was carried out in a manner similar to that described by Crutchfield.[5] The distillation was performed according to the method of Willard and Winter.[6] Determination of the amount of fluorine in the distillate was made by a colorimetric method based upon the bleaching of the alizarin-thorium lake by fluorine in a monochloracetic acid buffer.[7] Readings were made in an Evelyn colorimeter with a 520 filter.

Results. The average weekly rates of growth for the first 6 weeks and for the entire 14-week period are presented in Table I. The rate of growth is expressed as growth of males as outlined by Schweigert *et al.*[4] In most cases where 90 p.p.m. or more fluorine were

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[7] Unpublished data.

TABLE I.
Effect of Fluorine Additions to the Ration upon Growth and upon Incidence and Extent of Carious Lesions in the Cotton Rat.

	No. of cotton rats	Avg growth/ week for 6 wks, g	Avg growth/ week for 14 wks, g	Avg No. of carious lesions	Avg extent of carious lesions	Avg No. of carious lesions in third molars	Avg extent of carious lesions in third molars
802 + 4% 1:20 L.E.	3	8.5	6.9	19.0	71+	4.3	12+
802 + 4% 1:20 L.E. + 22.5 p.p.m. F	3	8.5	6.6	16.3	57+	3.3	11+
802 + 4% 1:20 L.E.	5	10.8	7.0	27.2	85+	6.4	24+
802 + 4% 1:20 L.E. + 45 p.p.m. F	7	10.3	6.9	20.4	56+	4.7	13+
802 + 4% 1:20 L.E.	4	10.5	6.6	29.9	103+	7.0	27+
802 + 4% 1:20 L.E. + 90 p.p.m. F	7	8.9	5.5	10.3	21+	2.4	6+
802 + 4% 1:20 L.E.	3	9.0	5.9	33.7	102+	8.6	27+
802 + 4% 1:20 L.E. + 135 p.p.m. F	3	6.5	4.4	20.0	51+	3.0	6+
802	3	7.7	4.0	30.0	86+	7.6	24+
802 + 135 p.p.m. F	3	6.6	4.4	2.0	4+	0	0
802 + 4% 1:20 L.E.	3	10.0	6.6	28.0	97+	6.6	27+
802 + 4% 1:20 L.E. + 225 p.p.m. F	5	9.8	6.2	2.6	7+	1.2	4+
802 + 4% 1:20 L.E.	2	10.9	6.8	28.0	94+	6.0	25+
802 + 4% 1:20 L.E. + 450 p.p.m. F	3	9.0	5.0	2.7	7+	2.0	5+

added to the basal ration (802 + 4% 1:20 liver extract), some inhibition in the rate of growth resulted, although in one series the growth rates for the 135 p.p.m. group and the 225 p.p.m. group were approximately the same as their respective controls. The rates of growth and caries incidence and extent in various control groups cannot be directly compared since different litters are represented in each group.

The inhibitory effect of fluorine, as sodium fluoride in the ration, upon dental caries formation is presented in Table I. It can be seen that the ingestion of 22.5 p.p.m. of fluorine did not result in a decrease in the incidence or extent of the carious lesions. However, 45, 90, and 135 p.p.m. of fluorine produced a progressive decrease in both the incidence and extent. At these levels the carious lesions observed were darker in color and not as soft as those observed in the animals on the basal ration without added fluorine. The ingestion of 225 or 450 p.p.m. of fluorine produced the maximum inhibitory effects.

The crowns of the third molars are not completely formed when the cotton rat is weaned. Therefore the third molars of the cotton rats which received fluorine in these experiments were partially formed in the presence of fluorine. No toxic effects were observed in the third molars of those animals which received less than 135 p.p.m. of fluorine in the ration. When 135 p.p.m. were fed, the enamel of the third molars was slightly mottled. At 225 p.p.m. there was a severe mottling while at 450 p.p.m. the mottling was most extensive and some enamel hypoplasia was observed.

In order to determine whether fluorine had a greater inhibitory effect on the occurrence of carious lesions in the third molars, the incidence and extent of carious lesions in these molars were calculated since they were formed during fluorine ingestion. When 45, 90, or 135 p.p.m. of fluorine were fed, the results indicated that the reduction in the incidence and extent of caries in the third molars closely paralleled the reduction in the first and second molars. When 225 or 450 p.p.m. of fluorine were fed, the incidence and extent of carious lesions in the third molars were higher than in the first and second molars.

A slight inhibitory effect of fluorine on the formation of the pigment in the enamel of the incisors was observed when 45 p.p.m. of fluorine were fed. At this level the lower incisors developed a few, irregular white or pale areas. When 90 p.p.m. of fluorine were fed, the enamel of the lower incisors was almost completely white but that of the upper incisors was still practically normal in color. When 135 or 225 p.p.m. of fluorine were fed, the enamel of the incisors was a uniformly, pearly white, whereas the enamel of the incisors became very chalky with a complete loss of the normal opalescence when 450 p.p.m. of fluorine were ingested.

Analyses of the dextrin and sucrose used in previous carbohydrate experiments indicated fluorine contents of 3 to 4 p.p.m. and 1 to 2 p.p.m., respectively. Therefore, the inhibitory effect of dextrin upon the formation and development of carious lesions was not due to the fluorine content.

Discussion. Miller[1] observed a 90% inhibition of experimental dental caries in the white rat when the rats were given the caries-producing diet plus 250 mg of sodium fluoride per kilogram and drinking water containing 4.2 mg of sodium fluoride per liter. McClure and Arnold[8] reported that the production of dental caries in the white rat was significantly reduced by 125 p.p.m. of fluorine, as sodium fluoride, in the food. Hodge and Finn[9] and Cheyne[10] have reported that the ingestion of 3 mg of fluorine as potassium fluoride decreased the incidence of caries production by 80-85%. This level is approximately twice that shown to be effective by Miller[1] and McClure and Arnold.[8] The levels of fluorine fed by the latter authors[1,8] are approximately comparable to 135 p.p.m. of fluorine in the ration. When 135 p.p.m. of fluorine were fed to cotton rats, as an addition to the basal ration, 802, an inhibition of over 90% was produced. When 135 p.p.m. of fluorine were added to the basal ration which contained liver powder, 802 + 4% 1:20 liver extract, only about 40% inhibition was produced. However, this was possibly due to an increased susceptibility of this particular group to tooth decay since the inhibition observed was not as great as that produced by 90 p.p.m. of fluorine. Previously we have shown that the addition of 4% 1:20 liver extract had no effect upon the production of carious lesions.[2,3] This is also shown when the two groups of controls for the 135 p.p.m. of fluorine studies are compared. The levels of sodium fluoride fed by Hodge and Finn[9] and Cheyne[10] are comparable to about 225 p.p.m. of fluorine at which level 90% inhibition was observed in the cotton rat. Dixon and Cox[11] have reported that 250 p.p.m. of sodium fluoride in the ration produced mottled enamel in the third molars of weanling rats. Cheyne[12] has shown that as low as 150 p.p.m. of fluorine fed as potassium fluoride to day-old rats was sufficient to produce mottling in all molars. Cox *et al.*[13] observed mottling of the enamel of the first and second molars of white rats fed 128 and 256 γ of fluorine per day for the first 21 days of life. Mottled enamel of the third molars was produced in the cotton rat by as low as 135 p.p.m. of fluorine in the ration.

The level of fluorine in the ration of the cotton rat necessary to inhibit the production of carious lesions appears to be very similar to that in the white rat even though the method of producing the carious lesions was very different. The levels of fluorine at which fluorosis of the teeth occurs are also similar in the two species.

It was observed that the reduction of the incidence and extent of carious lesions in the third molars appeared to be the same as the reduction observed in the first and second molars. This is in contrast to previous reports that those teeth formed during fluorine ingestion in man[14,15,16] and in the white rat[17] were more resistant to tooth decay than those subjected to fluorine only after tooth development was complete. The reason for this difference is not known.

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[8] McClure, F. J., and Arnold, F. A., Jr., *J. Dent. Res.*, 1941, **20**, 97.

[9] Hodge, H. C., and Finn, S. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 318.

[10] Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 58.

[11] Dixon, S. F., and Cox, G. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 236.

[12] Cheyne, V. D., *J. Dent. Res.*, 1942, **21**, 145.

[13] Cox, G. J., Matuschak, M. C., Dixon, S. F., and Walker, W. E., *Science*, 1939, **90**, 83.

[14] Dean, H. J., Jay, P., Arnold, F. A., Jr., McClure, F. J., and Elvove, E., *U. S. Pub. Health Rep.*, 1939, **54**, 862.

[15] Dean, H. J., Jay, P., Arnold, F. A., Jr., Elvove, E., Johnston, D. C., and Short, E. M., *U. S. Pub. Health Rep.*, 1941, **56**, 761.

[16] Dean, H. J., Arnold, F. A., Jr., Elvove, E., Johnston, D. C., and Short, E. M., *U. S. Pub. Health Rep.*, 1942, **57**, 1155.

[17] Cox, G. J., Matuschak, M. C., Dixon, S. F., Dodds, M. L., and Walker, W. E., *J. Dent. Res.*, 1939, **18**, 481.

Summary. Some inhibition of caries resulted when 45, 90, or 135 p.p.m. of fluorine were ingested as supplements to a sucrose basal ration. Depigmentation of the incisors appeared when 45 or more p.p.m. of fluorine were incorporated in the diet while mottling of the third molars occurred when 225 or more p.p.m. were fed. The ingestion of 225 or 450 p.p.m. of fluorine caused a 90% decrease in caries incidence. Thus the amount of fluorine necessary to inhibit caries to this degree was sufficiently high to cause mottling of the developing teeth.

The inhibitory effect of dextrin upon caries development could not be explained on the basis of its fluorine content.

14993

The A-V Conduction System in the Heart of the Guinea Pig.

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The importance of the conducting system in the heart is attested by increasing numbers of references to its structure and function.[1,2,3] Methods of study have involved gross dissection, injection with opaque substances, and histologic studies of serial sections of the heart.

The present work consists of a study of serial sections of young guinea pig hearts stained with Goldner's modification of the Masson trichrome technic.[4] The conducting tissue stains a purplish gray, the myocardium is brick red, and connective tissue is a bright green. In the guinea pig heart, there is not the slightest difficulty, even under low power of the microscope, in distinguishing A-V conducting tissue from ordinary myocardial tissue. Fig. 1 shows the clear-cut distinction between bundle tissue (B) and myocardial tissue (SE) of the interventricular septum. Under high power, the nodal and bundle cells are of the Purkinje type. The A-V node lies in the posterior part of the interatrial septum, as it does in other mammals. The main bundle and its right and left interventricular septal branches can easily be followed through the serial sections to their peripheral connections. The distribution of the right and left septal branches is similar to that described for other hearts. However, in this heart there are additional interventricular septal branches, one of which is shown in Fig. 1 (S). These additional branches descend directly from the main bundle. There are also connections to the septal muscle from proximal as well as distal parts of the right and left bundle branches.

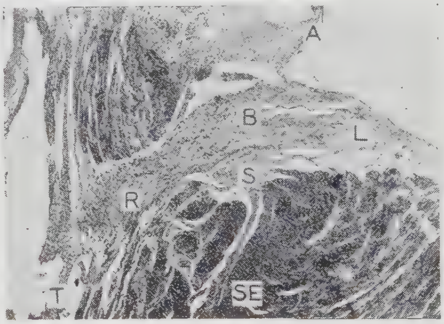

FIG. 1.

Photomicrograph of the main bundle (B) of the A-V conducting system, the septal branch (S), and the beginning of the right (R) and left (L) bundle branches in the interventricular septum (SE) of the heart of a young guinea pig. Semilunar valve of aorta (A), tricuspid valve (T). Frontal section, 10 μ thickness, Masson's trichrome stain.

These muscular connections of conducting tissue may have considerable significance with regard to analysis of function of heart muscle.

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[1] Ashman and Hull, *Essentials of Electrocardiography*, 1941, p. 28, 51, 69.

[2] Bayley, R. H., *Am. Heart J.*, 1943, **26**, 769.

[3] Nonidez, J. F., *Am. Heart J.*, 1943, **26**, 577.

[4] Goldner, J., *Am. J. Path.*, 1938, **14**, 237.

The electrocardiogram of a guinea pig is very similar to that of other laboratory animals. Therefore analytical studies of electrocardiograms must consider more carefully than heretofore the anatomical patterns of the conducting system.

Summary. In the guinea pig heart, the A-V conducting tissue is readily differentiated under the microscope from the myocardial tissue. The distribution of the right and left branches of the A-V bundle is similar to that described for other mammalian hearts. In addition there are other septal branches in this heart which are not seen in most mammalian hearts. The significance of these findings is pointed out.

14994

Vaccination with Various Western Equine Encephalomyelitis Viruses; Comparison as Antigens and as Test Inocula.*

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From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Vaccination of mice and guinea pigs with various strains of Western equine encephalomyelitis (W.E.E.) virus, carried out recently in this laboratory, induced a satisfactory degree of immunity to all except the current Rockefeller Institute (R.I.) strain. Indeed, mice immunized with the latter to such a degree as to show immunity to other W.E.E. viruses, failed to resist comparable amounts of the homologous R.I. strain.

Sabin reported[1] earlier that certain strains employed in his laboratory may possibly have different antigenic constitutions. Hammon cited[1] experiences similar to ours with respect to the use of current R.I. strains in challenge tests for immunity.

A study was undertaken to determine whether the various strains of W.E.E. virus which were available showed immunological differences; as a corollary, to select strains best suited not only for preparation of vaccines but also for use as standard in challenge tests for induced immunity.

The W.E.E. strains of the present investigation comprised the following:

The *R.I.* strain was obtained in 1933 from Miss B. F. Howitt, then at the George Williams Hooper Foundation, San Francisco. Since that time it has undergone frequent intracerebral passages in mice at the Rockefeller Institute. Three samples were available, hereafter referred to as (a) "R.I."—the virus currently used; (b) "R.I. 1939"; and (c) "R.I. 1941," the dates referring to the year in which the strains were frozen and kept so until required for use as fresh virus when an occasional mouse-passage was made.

The *Lederle* strain was sent to us in the form of guinea pig brain on Sept. 8, 1943, by Miss F. L. Clapp of the Lederle Laboratories, Pearl River, N.Y. It was in the 2nd guinea pig passage and had ultimately been derived from horse brain obtained by the Lederle Laboratories from the Bureau of Animal Industry.

The *Mosquito* strain was received as a mouse-brain passage of Strain F-199, on Oct. 19, 1943, from Dr. Hammon of the Hooper Foundation. It had been isolated by Dr. Hammon from *Culex tarsalis* in the Yakima Valley, Washington.

The *Kelser* strain was sent by Brigadier General R. A. Kelser, Chief of the Veterinary Division, U.S.A., to Dr. C. TenBroeck, who offered it to Dr. J. Zichis of the Illinois Department of Public Health, Chicago, and he in turn gave it to us in 1942. The strain has undergone many guinea pig passages and

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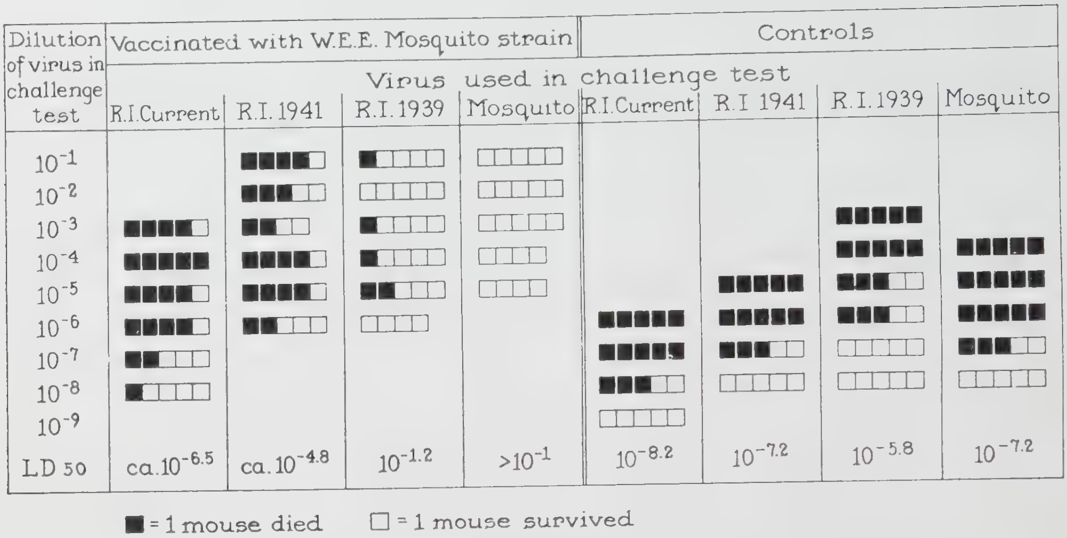
* This study was carried out under the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

[†] Captain, M. C., A. U. S.

[1] By personal communication.



Dilution of virus in challenge test	Vaccinated with W.E.E. Mosquito strain				Controls			
	Virus used in challenge test							
	R.I.Current	R.I. 1941	R.I. 1939	Mosquito	R.I.Current	R.I 1941	R.I. 1939	Mosquito
10^{-1}		■■■■□	■□□□□	□□□□□				
10^{-2}		■■■□□	□□□□□	□□□□□				
10^{-3}	■■■■□	■■□□	■□□□□	□□□□□			■■■■■	
10^{-4}	■■■■■	■■■■□	■□□□□	□□□□			■■■■■	■■■■■
10^{-5}	■■■■□	■■■■□	■■□□□	□□□□		■■■■■	■■■□□	■■■■■
10^{-6}	■■■■□	■■□□□	□□□□		■■■■■	■■■■■	■■■□□	■■■■■
10^{-7}	■■□□□				■■■■■	■■■□□	□□□□□	■■■□□
10^{-8}	■□□□□				■■■□□	□□□□□	□□□□□	□□□□□
10^{-9}					□□□□□			
LD 50	ca. $10^{-6.5}$	ca. $10^{-4.8}$	$10^{-1.2}$	$>10^{-1}$	$10^{-8.2}$	$10^{-7.2}$	$10^{-5.8}$	$10^{-7.2}$

■ = 1 mouse died □ = 1 mouse survived

GRAPH 1.

Intensive vaccination with mosquito strain and challenge tests with different samples of R.I. virus and with the homologous strain.

(The 1939 R.I. strain as used here was derived from frozen mouse-brain virus—as were the other viruses—and the prior two determinations of its LD_{50} titer were $10^{-7.6}$ and $10^{-7.3}$.)

was used earlier in this laboratory and described in studies on serotherapy.[2]

Method of Vaccination. Vaccines consisted of 10% virus-infected chick-embryo containing 0.1 to 0.4% formalin and were stored at 4°C. Different procedures of immunization were carried out in several series of repeated experiments; in one, mice received subcutaneously 0.1 cc of each of the vaccines to be tested twice at a week's interval, the total amount given being 0.2 cc. The test for immunity was applied about 14 days after the first dose. This is designated as a "light" type of immunization. In other procedures, more intensive vaccination ("intermediate" and "intensive") was carried out, *e.g.*, 2 courses of intraäbdominal or subcutaneous inoculations given on 3 consecutive days with a week's interval between the courses: the total amount of vaccine per mouse varied from 0.6 to 1.5 cc. Two weeks after the first dose the mice were tested for immunity over a range of serial tenfold virus (mouse-brain) dilutions given intracerebrally. Thus differing shades of response were elicited to differing degrees of immunization.

Demonstration of Strain Differences. Three typical experiments, each representing one of the immunization schedules described, are shown in Table I.

Results indicate that, no matter which strain served as vaccine, within each experiment the degrees of resistance to the Lederle, Mosquito, and Kelser strains were of the same order of magnitude. In contrast, resistance to the current R.I. strain was either not demonstrable or of much lower degree. The outcome of these and other similar experiments furthermore suggests that the Mosquito strain, *i.e.*, the one with the shortest laboratory history, may be somewhat superior to the others in immunogenic potency.

The following studies were undertaken in an attempt to determine any factor responsible for the failure of the current R.I. strain to indicate in full measure the immune state of a vaccinated animal.

Immunological Check on Identity of the Current R.I. Strain. The tests already described furnished evidence that the current R.I. strain was immunologically related to the other 3 strains of W.E.E. virus used. By immunizing mice with Eastern or Western inactivated virus and testing with Eastern or cur-

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[2] Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, **77**, 359.

TABLE I.
Response of Mice Immunized by Various Procedures to Several Strains of Virus.

Exp. 1. Immunity to intracerebral challenge dose of W.E.E. viruses after "light" type of immunization (2 doses each 0.1 cc vaccine subcutaneously).

Vaccinated with	Tested with active virus intracerebrally			
	R.I. (current)	Lederle	Mosquito	Kelser
R.I.	0;10*	0;10	0;10	10
Lederle	0;0	10;10	>10;0	10
Mosquito	0;0;10	1000;100	>10;0;10	100;10
Kelser	0;0	100;40	>10;0	100;10

Exp. 2. Immunity to intracerebral challenge dose of W.E.E. viruses after "intermediate" type of immunization (6 doses each 0.1 cc subcutaneously).

Vaccinated with	Tested with active virus intracerebrally			
	R.I. (current)	Lederle	Mosquito	Kelser
R.I.		0		
Lederle	0	10	>10	0
Mosquito	0	>1000	>10	>1000
Kelser	0	100	>10	100

Exp. 3. Immunity to intracerebral challenge dose of W.E.E. viruses after "intensive" type of immunization (6 doses each 0.25 cc intraäbdominally).

Vaccinated with	Tested with active virus intracerebrally			
	R.I. (current)	Lederle	Mosquito	Kelser
R.I.	$400;10^3$	$>10^5$	$>10^5$	$>10^5$
Lederle	$300;10^3$	$>10^5;>10^5$	$>10^5;>10^5$	$>10^5;>10^5$
Mosquito	$>4000;10^3$	$>10^5;>10^5$	$>10^5;>10^5$	$>10^5;>10^5$
Kelser	$600;10^3$	$>10^5;>10^5$	$>10^5;>10^5$	$>10^5;>10^5$

* 0;10 = Results of repeated tests. The figures represent the resistance index: ratio of titer in the vaccinated to that in the control mice.
> = more than the number of m.l.d. stated.

rent R.I. strain, it was found that the latter is a Western virus which shows no cross-reaction with the Eastern. This was also confirmed by Dr. Casals of this laboratory by means of active immunization of guinea pigs and by complement-fixation and neutralization tests.

The possible existence of strain-specific neutralizing antibody was tested by vaccinating 2 groups of mice, one with the R.I. (current) strain and the other with Kelser virus. After light type of immunization as described, a minimal amount of immunity to each strain was demonstrated in each case (undiluted serum neutralized 10 m.l.d. of virus by intracerebral test in mice). Thus no evidence was derived through vaccination to show strain-specificity for the 2 viruses.

Comparison of Subcutaneous and Intraabdominal Immunization. A Lederle-strain vaccine was injected into mice following the pattern of the intensive type of immunization. They were challenged by an intracerebral test of Lederle and of current R.I. viruses. Mice injected subcutaneously failed to resist 100 LD_{50} of the R.I. strain (smallest amount tested); those treated intraäbdominally resisted 300 LD_{50} of it. Mice vaccinated by either route and tested with the homologous Lederle strain withstood at least 10,000 LD_{50}. Although not all the tests showed end-points, no striking difference between intraäbdominal and subcutaneous routes of vaccination was demonstrable; the difference according to the strain used for the challenge dose was, however, marked: little or no immunity to the R.I. current strain yet solid immunity to the Lederle.

Changes in the R.I. Strain. Graph 1 illustrates one of repeated tests in which mice intensively immunized with a single strain (Mosquito strain) were challenged with the 3 available samples of the R.I. strain and with the homologous virus. To the latter, the Mosquito strain, the vaccinated showed complete protection; to the current R.I. strain, only to a slight degree, or 50 LD_{50}. A possibly

TABLE II.
Properties of the Various Strains.

Virus in form of infected	Host species and route	Property compared	Strain of virus			
			R.I. current	Lederle	Mosquito	Kelser
Mouse brain	Mice intracerebral	Titer (inverse log)	7.2-9.0	6.6-7.4	5.0-7.2	6.5-7.3
Whole chick embryo			7.5	7.5	7.5	7.5
Mouse brain	Mice intracerebral	Avg interval between inoculation and death — Virus dilution	10–1-10–7	10–1-10–5	10–1-10–5	10–1-10–5
		Days	2.5	5	5.5	5.5
Whole chick embryo	Chick embryo; toward embryo	Hours	20	20	40	20

somewhat higher degree of protection was exhibited by the mice tested with the R.I. 1941 sample, and almost complete protection by those challenged with the R.I. 1939 sample (Graph 1). The odd animal which succumbed in the latter group, died (with one exception in the 10^{-1} dilution) after a prolonged incubation period. Moreover, this unevenness of response to the 1939 virus may possibly be a reflection of the development of a change in this sample in a direction toward the type of reaction induced by the 1941 strain and even more strikingly by the current R.I. strain. As has been pointed out, the 3 samples of the R.I. strain differ in that between 1939 and 1941, and again to the present time frequent additional, consecutive, intracerebral passages in mice have been carried out. These passages may have brought about the change suggested by the relative difficulty encountered in immunizing effectively against the later samples. It is noteworthy that the incubation period after intracerebral inoculations in mice of the current R.I. strain is now 2 to 3 days as compared with that of earlier samples of 3 to 4 days, and that its titer is generally somewhat higher (LD_{50} ranging from $10^{-7.2}$ to $10^{-9.0}$) than the present titers of the 1941 ($10^{-6.8}$ to $10^{-7.8}$) and 1939 ($10^{-5.8}$ to $10^{-7.6}$) samples. Table II shows certain properties of the current R.I. strain in comparison with those of other W.E.E. viruses.

It is of interest in this connection that Burnet[3] concluded from his experience that influenza virus, well-adapted to growth in the allantoic cavity of chick embryos, after 42 and 60 passages, is more difficult to neutralize by antiserum than virus with only a few passages (4 and 1, respectively) in this medium.

Conclusions. (1) The immunological identity of 4 strains of W.E.E. virus was confirmed by tests in mice. (2) Results of comparative tests for their antigenic potency suggested that the one most recently isolated may be somewhat superior to the older laboratory strains. (3) Mice vaccinated with comparable doses of either of the 4 strains showed equal degrees of resistance to 3 of the strains, while tests with the 4th, the current R.I. strain, indicated that this strain is not a sensitive indicator of the degree of immunity of vaccinated mice. (4) Parallel tests in which vaccinated mice were challenged intracerebrally with the current and with earlier samples of the R.I. strain indicate that after its 9th or 10th year of propagation in laboratory mice, certain changes may have taken place which rendered this strain increasingly less suitable for use in challenge tests for immunity.

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[3] Burnet, F. M., *Australian J. Exp. Biol. and Med. Sci.*, 1943, **21**, 231.

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